Black and White: Investigations on the ecology, biology and biological control of sapstain fungi in New Zealand

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Abstract

This study examined ecology, colonisation, biological control and detection of sapstain fungi in New Zealand.

A nationwide survey of New Zealand sapstain fungi was undertaken between 1996 and 1998. In total, 1035 sites were sampled in the North and South Islands. From these collections, 1755 potential sapstain fungi were isolated and seventeen individual species were identified. The predominant sapstain fungi identified were *Sphaeropsis sapinea, Ophiostoma ips, O. floccosum, O. piliferum, O. querci and Leptographium procerum.* This data was subsequently evaluated according to ecological criteria, including geographical and temporal distribution of sapstain fungi. A greater diversity of sapstain fungi was found in areas of New Zealand that were primarily plantation forestry. *S. sapinea* was isolated during more generalised forest sampling, and from both native and plantation forests. *Ophiostoma* species were isolated more frequently from *Pinus radiata* plantations, processing plants including mills and ports and were mainly isolated from harvested forest material such as logs, wood chips, and other wood products. *S. sapinea* was isolated more frequently in spring and summer while *Ophiostoma* species predominated during autumn and winter periods, probably linked with insect vectoring. This was the first attempt to link the sapstain organisms identified in New Zealand with their overall distribution within sectors of the forest industry, in different wood species, and in different types of wood samples.

New Zealand is one of the world's largest exporters of softwood logs. Exports of forest products provide 4% of New Zealand's gross domestic product (GDP) with Japan, Korea, United States and the Philippines as major export markets for *P. radiata* logs. Conditions found within the holds of ships were thought likely to be ideal for the rapid colonisation of the sapstain fungi that results in dark penetrating stains. Log export research was designed to determine the extent of sapstain colonisation at specific points in the processing of logs from harvesting to the export destination. Two trials were established, in New Zealand summer and in winter, where mature *P. radiata* logs were harvested in New Zealand and shipped to export ports in Japan. Microclimatic conditions (temperature and relative humidity) on board the ships were recorded using data loggers both above deck and below deck. Nine species of sapstain fungi were isolated from logs during the summer trial. The most common species isolated were *O.*

floccosum, *O. querci* and *O. setosum*. In contrast, a different combination of nine species was detected during the winter trial. *S. sapinea*, *O. querci*, *O. floccosum*, *O. setosum* and *Ophiostoma piceae* were most commonly isolated. The results of this study showed that the New Zealand sapstain fungi were being exported on logs. However, many of these fungi were previously recorded in Japan. In addition, no serious pathogens were detected.

It was recognised during the sapstain fungal survey and export trials that a more thorough fundamental understanding of sapstain colonisation was required to properly determine the impacts of sapstain fungi to the New Zealand forest industry. *In vitro* and field studies were established to measure the relative colonisation of different species of sapstain fungi using synthetic media and *P. radiata* wood. These studies were undertaken to determine which sapstain species colonised, and how species interacted with each other following inoculation. In addition, the impacts of environmental conditions on the establishment and development of fungi and the resulting associated stains were determined. Temperature was found to affect the growth of various species of sapstain fungi on synthetic media and on *P. radiata* wood and logs. In the field studies *O. floccosum* was found to be the most successful coloniser of *P. radiata* logs in both winter and summer. Environmental factors such as temperature and moisture content were found to affect stain development. All fungal treatments developed sapstain discolouration from 15 days following inoculation in the summer field trial. In contrast, no stain was evident on any treatment in the winter field trial 60 days following inoculation. The colonisation of *P. radiata* by sapstain fungi was determined using microscopic techniques. Sapstain fungal hyphae were abundantly present in the ray parenchyma cells, tracheid lumen and resin canals of *P. radiata* wood. No differences in hyphal penetration were observed between the different sapstain fungal species at the cellular level. From these results a better understanding of the biology of sapstain species emerged. This new knowledge of the mechanisms and environmental triggers of fungal colonisation and stain development will aid in the more advanced technologies and management strategies to successfully control sapstain.

The many negative environmental impacts associated with traditional anti-sapstain chemical treatments led to increased interest in the more benign treatments of logs and wood using biological control technologies. The potential biological treatment of sapstain fungi using albino strains of *O. floccosum*, *O. piceae* and *O. pluriannulatum* was evaluated. These albino fungi demonstrated varied growth characteristics at different temperatures on both synthetic media and on *P. radiata* wood specimens. Comparable growth was found when evaluating wild type and albino sapstain strains in synthetic media and *P. radiata* wood in laboratory and field studies. Four field trials showed considerable variation in the biological control potential of the albino strains against wild type sapstain fungi. The mode of action of the albino strains was found to be competitive, through the use of primary resource capture of wood nutrients. However, albino *O. floccosum* strains were shown to produce zonal barriers when challenged with *S. sapinea*.

Proteomic analysis of cell wall proteins was undertaken to differentiate sapstain fungi from other wood inhabiting fungi and in an attempt to develop a method of determining the presence of sapstain fungi in wood prior to the development of stain. Proteins were extracted from the cell walls of various sapstain fungi (*L. procerum, O. ips, O. querci* and *S. sapinea*) and wood inhabiting fungi (*Alternaria alternata*, *Epicoccum nigrum*, *Trichoderma koningi*) grown in liquid culture. Two dimensional (2D) protein profiles were made and compared. A total of 93 proteins were analysed using mass spectroscopy and the profiles compared to protein databases. This is the first investigation using proteomics of the cell wall proteins of *Ophiostoma* species and *S. sapinea*.

This study addressed the importance of sapstain fungi in New Zealand on *P. radiata*, and increased our understanding of ecological requirements of the different sapstain species. Differences between these species with respect to colonisation and the development of associated stains were identified and investigated, enabling a critical evaluation of those species that were found to be most significant to the New Zealand forest industry. The future of sapstain management depends on a thorough ecological understanding of key sapstain fungal species involved in the cosmetic degradation of wood and an ability to manage the impacts of these species on the New Zealand forest industry using a combination of forest management practises and biological and chemical control techniques.

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To all my friends old and new, especially Joye, Mary, Liam, Kate and Rachael, thank you for making me who I am and supporting me in my crazy quest to be "Dr. Jo". You all have kept me inspired and sane and I thank you as well for just believing in me.

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List of species described in thesis research

The following is a list of first the tree species from which fungi were isolated, and second the formal fungal names and naming authorities for genus and species for the identified cultures and other cultures used in this thesis research. The mycological references these names and authorities are given in the text where the organism is first mentioned

Trees species
Acer negundo L.
Chamaecyparis lawsoniana (A. Mur.) Parl.
Cupressus lusitanica Miller
Dacrydium cupressinum Soland
Liriodendron tulipifera L.
Magnolia grandiflora L.
Metrosideras umbellate
Nothofagus menziesii (Hook.)
Nothofagus solandri var. solandri Derst.
Pinus radiata (D. Don)
Pinus taeda L.
Pinus nigra Arnold
Pseudotsuga menziesii (Mirb.) Franco

Fungal Genus and anamorphs
Alternaria Nees
Botryosphaeria Cesati & De Notaris
Ceratocystis Ellis & Halstead
Ceratocystiopsis Upad. & Kendrick
Chalara Corda
Cladosporium Link
Epicoccum Link
Fusarium Link
Geotrichum Link
Leptographium Lagerb. & Melin
Ophiostoma H. & P. Sydow
Penicillium Link
Pesotum Crane & Schoknecht
Pestalotia de Not
Sporothrix Haktoen & Perkins
Trichoderma Pers.
Verticillium Nees

Fungal species
Alternaria alternata (Fries) Keissier
Cladosporium cladosporioides (Fresenius) de Uries
Epicoccum nigrum Link
Leptographium procerum (Kendrick) Wingfield
Leptographium truncatum (Wingfield & Marasas) Wingfield
Ophiostoma coronata Olchow. & Reid
Ophiostoma floccosum Mathiesen
Ophiostoma galeiformis (Bakshi) Math-Käärik.

Ophiostoma huntii (Robinson-Jeffrey) de Hoog & Scheffer
Ophiostoma ips (Rumbold) Nannf.
Ophiostoma nigrocarpum (Davidson) de Hoog
Ophiostoma piceae (Münch) H & P. Sydow
Ophiostoma piliferum (Fries) H. & P. Sydow
Ophiostoma pluriannulatum (Hedgcock) H & P. Sydow
Ophiostoma querci Georgevitch Nannf.
Ophiostoma setosum Uzonovic, Seifert, Kim and Breuil
Ophiostoma stenocerus (Robak) Melin & Nannf.
Pesotum fragans Okada & Seifert
Sphaeropsis sapinea (Fr.:Fr) Dyko & Sutton
Trichoderma koningi Oudem

1 General introduction and literature review

1.1 Introduction

Sapstain is the discolouration of wood caused by the presence of pigmented hyphae, belonging to various taxonomic groups of fungi. This cosmetic discolouration to the wood imparts no loss of strength, but affects domestic and export earnings for the forest industries. The primary wood species in New Zealand, *Pinus radiata*, is highly susceptible to sapstain damage, with an estimated annual loss in revenue of NZ$100 million (Wakeling, 1997). Sapstain in New Zealand is principally caused by members of the Ophiostomataceae family and by *Sphaeropsis sapinea.*

In New Zealand, there are around 1.7 million hectares of plantation forestry and of this approximately 1.6 million consists of *P. radiata* (New Zealand Forest Owners, 2001). The fast growth of *P. radiata* in New Zealand results in the production of trees with a high proportion of sapwood, compared to many Northern Hemisphere softwoods (Cown, 1992). This increased sapwood imparts more susceptibility to detrimental fungi including mould, sapstain, decay and soft rot (Butcher and Drysdale, 1991) than is observed in Northern Hemisphere softwood species. This thesis research was concerned with the fungi that cause sapstain, which result in loss of value for logs and timber of New Zealand wood species, in particular *P. radiata.*

Little is known about the ecology and biology of the fungi involved in sapstain formation. "The lack of knowledge about the organisms causing timber discolouration (or stain) posed relatively few problems while sodium pentachlorophenol was in use" stated Kang and Morrell (2000). International concern and awareness of the health and environmental impacts of sodium pentachlorophenol and its derivates resulted in the discontinuation of this method of sapstain control. In order to develop an integrated management strategy or an environmentally benign technology against the stain, the ecology and biology of the staining organisms firstly needs to be well understood.

Historically, in New Zealand, there was limited research on sapstain fungi affecting *P. radiata.* The major sapstain problems of *P. radiata* were linked with *S. sapinea* (Birch, 1936; Butcher, 1967). Other minor sapstain species were also found, including members of the *Ophiostoma* family (Butcher, 1967). Hutchison and Reid (1988a, b) sampled wood from indigenous and native trees in six locations in the North Island and found a number of *Ophiostoma* species associated with sapstained wood. A more comprehensive survey of sapstain fungi in New Zealand was conducted from 1996 until 1998 (Farrell *et al*, 1998). The sapstain fungi isolated and identified in the study by Farrell and colleagues included *S. sapinea* and thirteen members of the Ophiostomataceae family. In this doctorial thesis, aspects of ecological distribution of sapstain fungi in New Zealand were investigated using the survey data of Farrell *et al* (1998). In addition the ecology and colonisation of sapstain fungi from harvesting to delivery at an export destination (Japan) was followed in two field trials. These trials were the first successful attempts published in the world, of sampling logs at harvesting, at the departure port before shipment and at the destination port in Japan. Besides the ecological significance for sapstain control, this thesis research had direct implications to biosecurity.

During this ecological investigation of New Zealand sapstain fungi, a number of *Ophiostoma* species and their anamorphs were described in New Zealand for the first time. These new species as well as previously studied species were included in more specific physiological research, which is described in this thesis. Physiological aspects of a selection of New Zealand sapstain fungi including factors controlling colonisation on synthetic media, *P. radiata* in the laboratory and *P. radiata* in the field were undertaken. An understanding of the factors influencing the colonisation and growth of sapstain is expected to provide valuable information to help in the development of more effective control and detection methods.

An albino *Ophiostoma piliferum* strain lacking the melanin-like compounds responsible for the discolouration of wood was developed in the late 1980's (Blanchette *et al*, 1992; Brush *et al*, 1994). In recent years new albino strains were developed from other *Ophiostoma* species specifically for the New Zealand forest industry (Held *et al*, in press). This thesis describes in vitro and field investigations into the colonisation of albino *Ophiostoma piceae*, *Ophiostoma floccosum* and *Ophiostoma pluriannulatum* strains. Field trials were also established to investigate the biological control potential of these new species of albino strains.

There is a requirement by the forest industry for rapid and reliable techniques to identify sapstain fungi and methods to differentiate them from moulds and other wood inhabiting fungi. This thesis describes the development of a method of identification and detection of sapstain fungi. An investigation of the cell wall proteins of sapstain fungi and other common wood inhabiting fungi using proteomic technology to identify specific marker proteins at a species or group level was undertaken. It was proposed that antibodies to specific marker proteins could be used as a potential detection method.

1.2 Hypothesis, Aims and Objectives

The hypothesis of this thesis research was that an understanding of the fungal species that cause sapstain, their distribution and colonisation in *P. radiata* in New Zealand was required in order to establish efficient control regimes for the fungi.

The three primary aims of this research were i) to determine the effect of the sapstain fungi, *Ophiostoma* species and *S. sapinea*, on New Zealand wood species and wood products, especially *P. radiata*, ii) to investigate biological control methods to minimise sapstain growth and stain development and iii) to establish potential methods of detection of sapstain fungi.

The primary objectives of this thesis were as follows:

- Examine the distribution in New Zealand of sapstain fungi. Firstly analyse survey data to identify the geographical, seasonal distribution of sapstain fungi. Secondly, to investigate the influence of wood type (chip, log, timber and seedling) and different sectors of the forestry industry (native forests, *P. radiata* plantations, other wood species plantations, mills and ports) on the distribution of sapstain.
- Assess the sapstain development and identify the sapstain fungi in two ecological investigations from harvesting in New Zealand to delivery of export logs in Japan.
- Identify the growth characteristics of the major sapstain species *in vitro* and in field trials on *P. radiata*. Examination of the type and intensity of stain produced on *P. radiata* of New Zealand sapstain fungi.
- Develop a greater understanding of the biology and control potential of the albino *Ophiostoma* strains developed.
- Identify a method of identification of New Zealand sapstain fungi to species level that does not require complex morphological examination.

1.3 Overview of thesis organisation

This thesis consists of eight chapters. In Chapter 1, the literature with regard to New Zealand forestry and *P. radiata* is reviewed as well as an introduction to wood inhabiting fungi and in particular sapstain fungi. The taxonomy, ecology of sapstaining fungi is reviewed to provide essential background knowledge on the mycological aspects of the research topic. The literature relating to more specific aspects of the research objectives are provided at the beginning of the individual chapters. Chapter 2 contains the descriptions of the general materials and methods used in this thesis. More specific descriptions of methods related to individual aspects of the research are provided in the material and method section in the appropriate chapters. The ecology of sapstain fungi in New Zealand is presented in Chapter 3. The distribution of sapstain fungi from harvesting to an export destination as well as consideration of the export shipping environment is described in Chapter 4. Analysis of the colonisation of a selection of sapstain fungi *in vitro* and the field trials is presented in Chapter 5. Chapter 6 describes the biological control of sapstain on *P. radiata* using albino strains of *O. floccosum*, *O. piceae* and *O. pluriannulatum*. The mode of action of the albino strains is also investigated in this chapter. Chapter 7 describes the development of one method of identification and detection using proteomics. A general summary and discussion of the data presented in this thesis is provided in Chapter 8 as well as recommendations for future research. The appendices include transcripts of the candidate's publications that are published or in press at the time of submission of this thesis.

The naming authorities for the identified cultures and other cultures used in this thesis are given in the list of species (page xvii).

1.4 Literature Review: Introduction to *Pinus radiata* and wood fungi

In this section, literature of the wood-fungal system is reviewed, with emphasis on the dependence of the fungi on the wood as their host. Firstly, literature on the development of the New Zealand forest industry with particular emphasis on the establishment of *P. radiata* plantations and the structure, composition and chemical properties of *P. radiata* is provided. An introduction of wood inhabiting fungi, in particular the literature relating to sapstain fungi is addressed in the second part of this literature review. Literature reviews relating to more specific aspects of the research objectives are provided at the beginning of the appropriate chapters. Literature emphasising factors influencing colonisation of wood by sapstain fungi is reviewed in Chapter 5. Literature relating to general control methods, biological control, and albino development are reviewed in Chapter 6. A review of the literature on various detection methods for sapstain fungi and an introduction to proteomics is provided in Chapter 7.

1.4.1 *Pinus radiata* (D. Don)

1.4.1.1 *Pinus radiata* in New Zealand

Indigenous forests once covered 80% of New Zealand. Native forest is now set aside for conservation purposes and New Zealand must rely on 1.7 million hectares (6% total land area) of planted production forest for the supply of forest products for domestic consumption and exports (New Zealand Forest Owners, 2001). Planted production forests in New Zealand are composed of 90% *P. radiata,* 5% Douglas fir (*Pseudotsuaga menziesii*), 2% other exotic softwoods and 3% exotic hardwoods (including *Eucalyptus* species) (New Zealand Forest Owners, 2001).

From the onset, the planting of trees in New Zealand for forestry purposes was almost exclusively based upon exotic tree species introduced into the country (Kirkland and Berg, 1997). It is unclear how and when *P. radiata* was first introduced into New Zealand, however by 1865, it was firmly established (Harris, 1991). In the 1870's, new settlers to New Zealand were planting *P. radiata* as single species shelterbelts and woodlots in both the North and South Islands (Harris, 1991). Experimental plantings were made in state owned forests in the Central North Island in the early twentieth century. The Royal Commission on Forestry in 1913 recommended *P. radiata* as the most suitable of the introduced tree species for extensive plantings by the state due to the ease of propagation, rapid height growth, high volume production and adaptability to a wide range of sites (Harris, 1991). In contrast, native species were considered more difficult to propagate and too slow growing to fit commercial plantation objectives (Kirkland and Berg, 1997).

P. radiata is native to three locations on the central coast of California (Cambria, Monterey, Ano Nuevo) and to two small Mexican islands (Guadalupe and Cedros) (Figure 1.1) (Kirkland and Berg, 1997). Native stands cover only 8,000 hectares (Kirkland and Berg, 1997). The first seed introductions of *P. radiata* into New Zealand were imported in the 1850's to 1880's from Monterey and Ano Nuevo (Maclaren, 1993; Kirkland and Berg, 1997). In the 1920's and 1930's, seed was collected from New Zealand shelterbelts, for the major forest plantings. As so much seed was required during this planting boom, there was little incentive or opportunity for seed suppliers to select better seed trees (Kirkland and Berg, 1997). Testing of the seedlots from the early New Zealand *P. radiata* populations showed faster growth in New Zealand sites than those collected directly from native stands (Kirkland and Berg, 1997). This improvement in cultivation in only a few generations was attributed to more outcrossing in plantations than in native wild stands and to the apparent mixed origin of New Zealand *P. radiata* (Kirkland and Berg, 1997).

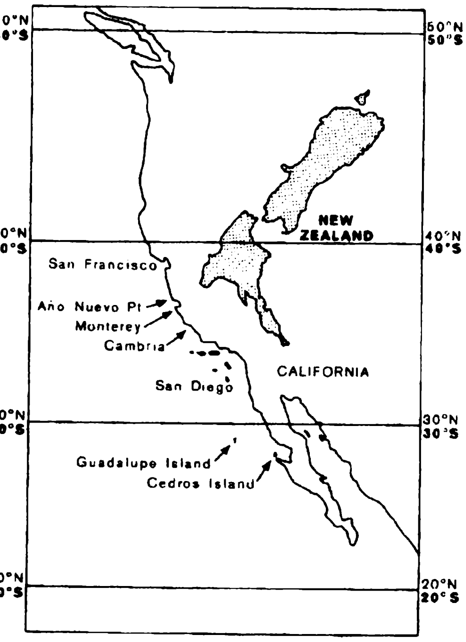


Figure 1.1: Locations of natural populations of *P. radiata* and equivalent latitudes to New Zealand (source Harris, 1991).

In New Zealand, *P. radiata* has a reputation for rapid growth under a wide range of growing conditions and an ability to meet diverse end-use requirements (Cown, 1992).

The yield and timber quality are a direct result of the interaction of a number of factors – site, silviculture and seedlot (Cown, 1992). Better selection of seed trees and the development of specialist seed orchards based on genetically improved trees, have improved stem straightness, tree form, vigour and wood density (Harris, 1991). In the 1980s, a system was developed in New Zealand that provides comparative rankings of genetic gain across a seedlot. There are four breeds, each with its own improvement rating: GF (growth and form), LI (long internode), DR (Dothistroma resistant) and HD (high wood density) (Maclaren, 1993).

P. radiata is planted widely throughout New Zealand from the north of the North Island to the south of the South Island, covering areas with different climate (from warm to cold) and different soil type (sand, pumice-scoria, and various types of clay) (Figure 1.2). The majority of the plantation forest area is situated in the Central North Island with 575,607 hectares (Figure 1.2). This thesis research utilised *P. radiata* logs and wood originating from Kinleith Forest within the Central North Island. The Central North Island soils are derived predominantly from volcanic ash from numerous eruptions over the last 20,000 years (New Zealand Soil Bureau, 1968). The thickness of the volcanic ash compaction, differences in nutrient levels as well as geographic features such as slope and aspect, contribute to most of the variation in soils (Rijske, 1994). Yellow brown pumice soils are the most extensively and frequently occurring soils in the forests of the Central North Island and are formed from pumice from the Taupo eruption (between 500 and 5000 years ago) (New Zealand Soil Bureau, 1968). The factors that may limit tree growth in these forests are a cool climate at higher altitudes and physical barriers to root growth (Rijske, 1994). The Central North Island region has an average annual rainfall of approximately 1,600mm (Quayle, 1984). The highest rainfall occurs during May to August, while the driest conditions are generally between November and February (Quayle, 1984). The prevailing winds are west to southwesterly. Temperature variations (both seasonal and diurnal) are relatively small due to New Zealand being a small landmass surrounded by ocean. Mean daily maximum temperatures are over 20°C during the summer period for the Central North Island. The mean temperature during winter months for this region is approximately 8°C. This area experiences approximately 1,950 sunshine hours per annum (Quayle, 1984).

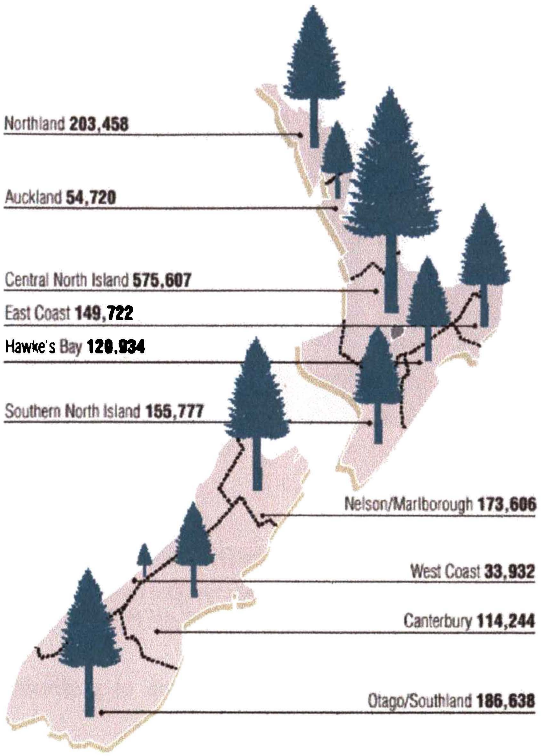


Figure 1.2: Map of New Zealand Forest plantations according to areas (hectares) (as of April 2000) (source New Zealand Forest Owners, 2001).

New Zealand's *P. radiata* plantations provide for almost all domestic wood needs, and also for a substantial export trade in logs and processed wood products. Of the commercial plantation forests in New Zealand to the year ending 31 March 2000, 35,000 hectares of forest area was clear felled with a total volume of 17,774,000 m^3 removed and the average clear fell age for *P. radiata* was 26.8 years. Harvested forest area is divided into three types of wood product; pulp logs, quality logs (saw logs, peeler logs and export logs) and other roundwood (small logs and export chips). The proportion of each wood product produced in New Zealand from 1994 - 2001 is shown in Figure 1.3.

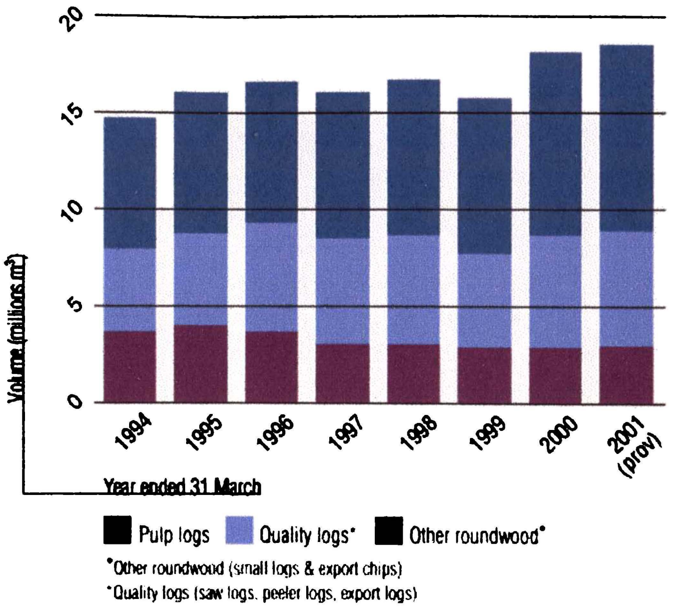


Figure 1.3: Harvested logs according to quality in New Zealand (source New Zealand Forest Owners, 2001).

1.4.1.2 Wood structure and composition of *P. radiata*

The structure of *P. radiata* is similar to other pines. Anatomically, *P. radiata* cannot be distinguished with certainty from *P. contorta* (lodgepole pine) and *P. ponderosa* (ponderosa pine) without the use of high power microscopy (Harris, 1991). Identification, however, is achieved by the degree of dentelation that develops on the inside of tracheids and the appearance of cross field pits.

P. radiata is comprised of three major cell types (tracheids, parenchyma and epithelial cells), which are arranged into axial cells (longitudinal orientation and contribute to wood strength) and ray cells (transverse orientation and involved in nutrient storage, transport and wood swelling) (Figure 1.4). The axial cells consist primarily of tracheids and account for 95% of *P. radiata* volume (Harris, 1991). In contrast, axial parenchyma cells are sparse in *P. radiata* (Harris, 1991) so the bulk of parenchyma cells are contained in the radially orientated wood ray cells.

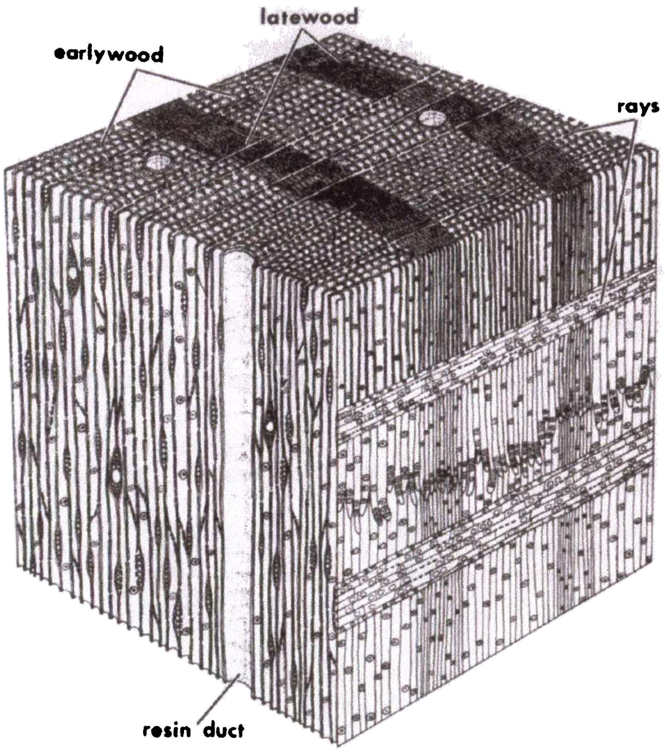


Figure 1.4: Block of softwood showing rays cells, resin ducts and the earlywood and latewood tracheids (source Eriksson *et al*, 1990).

Tracheids of the earlywood and latewood vary in wall thickness. Tracheids formed during the early spring (earlywood tracheids) are relatively large in diameter with thin walls and function in water and nutrient transport. Latewood tracheids have thicker walls, which are smaller in diameter, and contribute to wood strength. The contrast between earlywood and latewood gives rises to the appearances of annual rings seen in a log cross section (Harris, 1991). Sap flow in softwood tracheids occurs from cell to cell through intertracheary-bordered pits. These pits are specialised openings in the radial sidewalls of each tracheid.

Much of the parenchyma cells in the sapwood remain alive and are not lignified, acting as a food store, mainly for starch as well as soluble sugars, proteins, peptides and amino acids, lipids, nucleic acids and vitamins (Hudson, 1986). Once the tree is dead, these provide nutrient substrates for a variety of fungi. Half bordered pits are present in cell walls between axial tracheids and radially orientated parenchyma cells.

Resin canals are most frequent in the transition zone between earlywood and latewood (Harris, 1991). Resin canals are found in most softwood species, and lie in both the radial and axial direction. The resin canals are the source of resin exudation in freshly felled trees. Resin canals consist of a central tube surrounded by short parenchyma cells. In the living tree the parenchyma cells secrete resin into these canals at a considerable pressure.

Each wood cell is composed of various layers (Figure 1.5). The middle lamella and primary wall forms the compound middle lamella, located between the secondary walls of adjacent cells. The secondary wall has three layers designated S_1, S_2 and S_3. S_1 is the outermost layer of the secondary wall, the middle layer is the S_2 region, which is usually the largest, and the S_3 is located nearest the lumen (Eriksson *et al*, 1990).

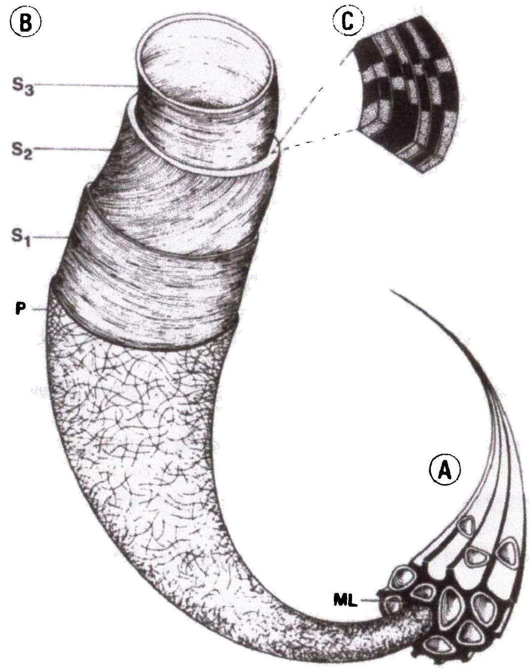


Figure 1.5: Structure of cell wall layers in tracheids (A) Tracheids, (B) cell wall layers, (C) arrangement of lignin and carbohydrates in the secondary wall. ML: middle lamella; P: primary wall; S_1, S_2, S_3: layers of the secondary wall (source Eriksson *et al*, 1990).

P. radiata is comprised of 40% cellulose, 31% hemicellulose, 27% lignin and 2% extractives (Uprichard, 1991). In *P. radiata*, as in other softwoods, cellulose is the principle component of the wood cell wall. Cellulose microfibrils constitute the structural framework of the tracheids and are surrounded and permeated by the cell wall matrix that is composed of lignin and hemicellulose. Cellulose is a linear polymer of β-1,4-linked anhydroglucose units.

The hemicelluloses are shorter chain polysaccharides, such as the arabinoglucuronoxylans, galactoglucomannans and arabinogalactans, which are composed of simple sugar monomers or related acidic compounds. Hemicellulose surrounds the cellulose microfibrils and occupies spaces between fibrils (Eriksson *et al*, 1990). The distribution of hemicellulose parallels that of lignin within the wall.

Softwood lignin is a three dimensional, heterogeneous, high molecular weight polymer of guaiacylpropane units. Although lignin contains hydroxyl groups, some of which are linked to the polysaccharides, it is a hydrophobic compound that resists swelling of the cell wall and provides rigidity to wood. Lignin is distributed throughout the secondary wall and compound middle lamella, but the greatest concentration is in the middle lamella (Eriksson *et al*, 1990).

The composition and distribution of *P. radiata* wood extractives often referred to as pitch, have been studied extensively. The resin content is low in comparison with other *Pinus* species, the main resin components being diterpene resin acids, fats, fatty acids, sterols and phenols (Porter, 1969). Hemingway and Hillis (1971) studied the distribution of these compounds and found that resin acids are concentrated in the inner heartwood, whereas sapwood contained a higher proportion of fatty acid esters. The extractive content increases considerably when sapwood is transformed to heartwood. In *P. radiata*, the source of resins are in the axially and radially orientated resin canals (which produce mainly resin acids) and the parenchyma cells (which produce the fatty acid esters, unsaponifiable materials and also a trace amount of free fatty acids) (Uprichard, 1991). The major resin acids in *P. radiata* are leuopimaric, palustric, pimaric, neoabietic and abietic acids. Uprichard and Lloyd (1980) determined the relative amounts of fatty acids (esters and free), resin acids, phenols and unsaponifiables in samples of sapwood and heartwood (Table 1.1).

Table 1.1: Compounds (% of total extractives) in heartwood and sapwood of *P. radiata* (source Uprichard and Lloyd, 1980).

Compounds	Heartwood	Sapwood
Fatty acids (free)	2	1
Fatty acids esters	11	41
Resin acids	71	41
Phenols	6	3
Unsaponifiables	10	14

Forest species in the Northern Hemisphere are dormant for several months of the year, and their annual growth cycle associated with the build up of carbohydrates during autumn and rapid depletion in late spring and summer when conditions favour growth. In contrast species such as *P. radiata* in New Zealand continue to grow throughout the year and the non-structural carbohydrates show no seasonal variation (Cranswick *et al*, 1987). Cranswick *et al* (1987) examined the seasonal variations of glucose, fructose, sucrose, cyclitols, quinic acid, shikimic acid and starch using ethanol extracts of increment core samples representative of one to four year old wood from 12 year old *P. radiata* trees and showed that soluble carbohydrates and cyclitols were present in wood tissue. The relative amounts of the soluble carbohydrate component isolated are given in Table 1.2. Starch and sucrose comprised two-thirds of the carbohydrate component. The shikimic and quinic acids are generally regarded as lignin precursors.

Table 1.2: Sugars, cyclitols and starches as a percentage of the total extractives of *P. radiata* (source Cranswick *et al*, 1987).

Major components	Compounds	% of total
Monosaccharides	Glucose, fructose	12.8
Cyclitols	Myoinositol, sequoyitol, pinitol, and pinpollitol	16.4
Acids	Shikimic and quinic acids	3.0
Disaccharides	Sucrose	34.3
Polysaccharides	Starch	33.5

1.4.2 Wood fungi – moulds and decay

Although this thesis focuses on sapstain fungi, there is a range of other detrimental fungi that interact with the sapstain fungi within the wood substrate. Wood fungi cause four main types of damage – mould, sapstain, decay or soft rot. Figure 1.6 shows a drawing of the effect of mould, sapstain, decay fungi and soft-rot on the wood cell wall.

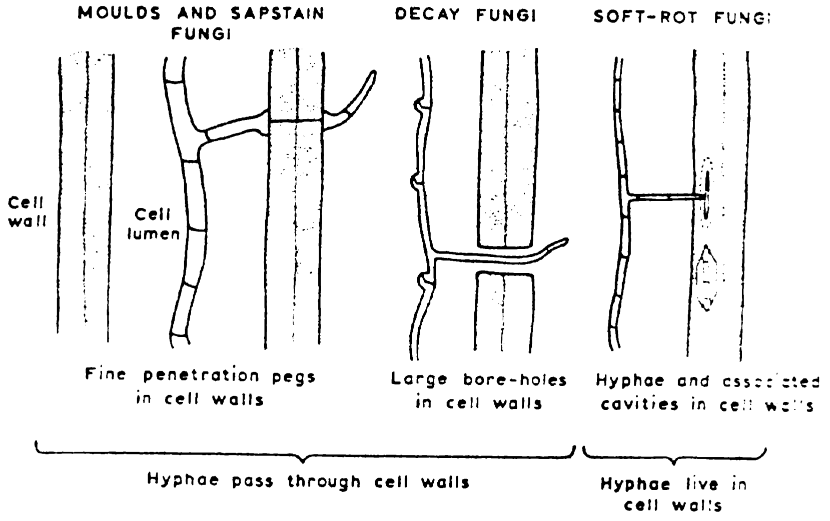


Figure 1.6: Effect of mould, sapstain, decay and soft rot on wood cell walls (source Butcher, 1974).

1.4.2.1 Mould fungi

The mould fungi are a large group of saprophytes, mainly Ascomycetes or Deuteromycetes. The production of coloured conidia results in the superficial discolouration of the wood that is generally of a woolly or powdery appearance (Butcher, 1974; Seifert, 1993). Mould fungi are also able to penetrate into the wood but as their hyphae are colourless their presence can only be detected by microscopic examination (Butcher, 1974). The presence of some mould fungi increases the permeability of wood allowing greater uptake of preservative chemicals but also greater reabsorption of water and thus a more favourable moisture content for colonisation of other wood fungi (Lindgren, 1952). They invade live wood more slowly than the sapstain fungi and prefer sapwood in which the medullary food reserves are still high (Verral, 1939). Common mould fungi isolated in New Zealand on wood include *Alternaria alternata*, *Aureobasidium pullulans*, *Epicoccum* species, *Trichoderma* species, and *Verticillium* species (Butcher and Drysdale, 1991).

1.4.2.2 Decay fungi

Decay fungi produce enzymes principally to enable them to utilise the components of the wood cell wall, cellulose hemicellulose and lignin. Mechanical strength is lost when the wood is colonised by decay fungi due to the degradation of cellulose and lignin. Three basic types of decay are recognised; white rot, brown rot and soft rot, each taking its name from the general appearance of decayed wood.

White rot fungi degrade cellulose, hemicellulose and most importantly the lignin component of the wood cell wall. The residual wood is typically fibrous with a whitish yellow to tan discolouration due to the removal of lignin. Most white rot fungi are Basidiomycetes, possessing dikaryotic hyphae and clamp connections along the septate hyphae. In contrast, brown rot fungi cause the extensive degradation of cellulose and hemicellulose and the modification, but not degradation, of lignin. The residue of heavily decayed wood (mainly modified lignin) easily crumbles and it is the inability of these fungi to degrade lignin that distinguishes them from white rot fungi (Eaton and Hale, 1993). Brown rot fungi produce expanding bore holes that are visible under the light microscope that assist in penetration into the axial cell system (tracheids) (Eriksson *et al*, 1990).

The term soft rot describes the surface softening of wood attacked by lignolytic Ascomycetes or Deuteromycetes. Soft rot decay results in wood of a spongy texture that when dried has an appearance similar to that degraded by brown rot fungi (Dix and Webster, 1995). Soft rot fungi are often described as primary colonisers of wood as they attack wood with relatively high moisture contents. Soft rot is characterised by chains of cavities in the S_2 cell wall layer. Fungal colonisation of the cell lumen is followed by the production of fine penetration hyphae into the S_2 layer. The release of degradation enzymes results in cavitation and localised loss of wood strength (Dix and Webster, 1995).

1.4.3 Sapstain fungi

Many fungi do not affect the integrity of the wood but may impart a discolouration or stain in the sapwood. The stain may be superficial or penetrate deeply into the sapwood, causing a stain that is blue, brown, red or a variety of colours. These fungi primarily colonise the parenchyma cells and utilize stored nutrients. Hyphae pass from one cell to another by growing through the pit membranes or directly through the cell wall. To penetrate the cell wall, the hyphal tip forms specialised structures called transpressorium. This constricted hyphae of small diameter moves into the wall and bores a hole through all cell wall layers. Once it has reached the lumen of the adjacent cell, the hypha resumes normal diameter.

Sapstain fungi are amongst the most economically important forest fungi and are distributed across a wide range of wood species (both hardwood and soft wood).
Seifert (1993) described the sapstain of wood as being caused by three groups of fungi;

- Species of *Ceratocystis*, *Ophiostoma* and *Ceratocystiopsis*
- Black yeasts, such as *Hormonema dematioides*, *Aureobasidium pullulans*, *Rhinocladiella atrovirens* and *Phialophora* species.
- Dark moulds, such as *Alternaria alternata* and *Cladosporium* species.

1.4.3.1 Fungal Melanin

Melanin is found in many fungi and is synthesised via at least four varieties of metabolic pathways (Zimmerman *et al*, 1995). Fungal melanin is composed of high molecular weight dark pigmented (generally black) phenolic polymers found in the cell walls of spores, mycelium or fruit bodies (Bell and Wheeler, 1986). Melanin was proposed to provide protection against various environmental threats including desiccation, ultra violet and visible light irradiation, fungicides and extremes of temperature (Bloomfield and Alexander 1967; Brasier 1978; Bell and Wheeler, 1986).

1.4.3.1.1 Melanin production

In the Ascomycetes and related Deuteromycetes, the dark-brown to black melanin in cell walls are generally synthesised via the pentaketide pathway and 1,8 dihydroxynaphthane (DHN) is the immediate precursor of the polymer (Figure 1.7) (Bell and Wheeler 1986). *Aspergillus niger* and most Basidiomycetes use other alternative pathways, possibly 3,4-dihydoxyphenylalanine (DOPA), glutaminyl-3,4-dihydroxybenzene (GDHB) or catechol (Wheeler, 1983).

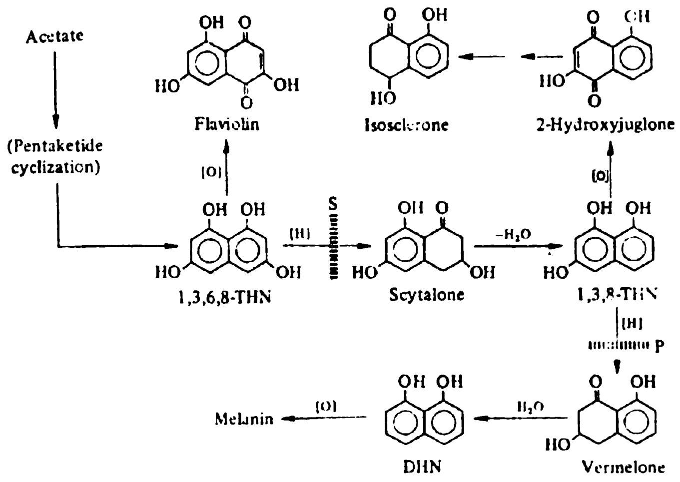


Figure 1.7: Pentaketide pathway of melanin biosynthesis for the Ascomycete fungi, *Vertiticillium dahliae*, *Pyricularia oryzae*, and *Thielaviopsis basicola*. 1,3,6,8-THN = 1,3,6,8-tetrahydroxynaphthalene; 1,3,8-THN = 1,3,8-trihydroxynaphthalene; DHN = 1,8-dihydroxynaphthalene (Source Wheeler, 1983).

The melanin of sapstain fungi was studied by electron microscopy and it was shown that the hyphae of two sapstain fungi (*C. coerulescens* and *A. alternata*) had deposits of melanin in the cell walls in the form of globular granules (Zink *et al*, 1989). Zimmerman *et al* (1995) found that *O. piliferum* produced melanin via DHN in penetaketide biosynthesis. Research by Eagen *et al* (1996) found genes from *A. alternata's* pentaketide pathway. They used a heterologous probe to screen southern blots from other sapstain fungi and detected the presence of homologous copies of the *A. alternata* DHN genes in the sapstain fungi, *O. floccosum* (Eagan *et al*, 1996). Further investigations by Eagen *et al* (2001) and Wang *et al* (2001) isolated and characterised the OSD1, a gene encoding scytalone dehydratase from *O. floccosum.*

Studies by Zimmerman *et al* (1995) showed that *O. piliferum* did not need melanin for growth and colonisation. Melanin however can influence survival and longevity, by protecting the fungi from UV light and desiccation (Eagen *et al*, 1996). The role of melanin has also been linked to enhancing competitive ability of the fungi (Zimmerman *et al*, 1995). Melanin is also needed for perithecium production. Zimmerman *et al* (1995) believed that melanin supports the development of elongated necks of the perithecium and dissemination of the ascospore droplets. In the absence of melanin only immature perithecia develop. After treatment with scytalone, a metabolite of the melanin pathway, melanin is synthesised and full perithecia development occurs (see Section 6.3.3 for more detail) (Zimmerman *et al*, 1995).

1.4.3.2 Historical background of sapstain research

Hartig, in 1878, was the first researcher to link discoloration of wood with darkly pigmented fungal hyphae, but limited the cause of discolouration to one sapstain fungus *Ophiostoma piliferum* (Seifert, 1993). In 1906, in North America, Hedgcock isolated *O. piliferum*, *O. pluriannulatum*, *O. minus* and *O. moniliformis* from lumber (Seifert, 1993). At around the same time in Europe, Münch isolated *O. piliferum*, *O. piceae*, *O. canum* and *O. minus* and demonstrated that they could discolour pine and spruce sapwood (Seifert, 1993). It was not until the early years of the twentieth century with the expansion of the forest and forest products industries that the economic importance of the sapstain fungi became evident (Wingfield *et al*, 1993). Much of the later work, especially on the *Ophiostoma* species was limited to taxonomic identification rather than consideration of the sapstaining quality of the fungi and an emphasis was placed particularly on the pathogenic species of the Ophiostomataceae family (Table 1.3).

The most comprehensive investigations were done on the pathogenic species of *Ophiostoma* and *Ceratocystis* – *O. ulmi* and *O. novo-ulmi*, the "Dutch Elm disease fungi" and *Ceratocystis fagacearum* the "oak wilt fungus" from the United States. *O. ulmi* is a weak pathogen on most European elm species, whereas *O. novo-ulmi* is characterised by high mortality rates among native European elms (Brasier, 1986; Brasier, 1991). *O. novo-ulmi* is further divided into two subgroups according to their respective geographical distribution and named Eurasian (EAN) and North American (NAN) races (Binz and Canevascini, 1996). There are also species of *Ophiostoma* that exhibit pathogenicity toward humans, notably *Sporothrix schenckii*, a conidial fungus causing sporotrichosis that has been linked to *Ophiostoma (*Berbee and Taylor, 1992). These species will be discussed along with the major groups causing sapstain in New Zealand in the sections 1.4.3.3 to 1.4.4.

Table 1.3: Some diseases caused by members of the Ophiostomataceae Family and their distribution (source Wingfield *et al*, 1993).

Fungus	Host	Disease	Distribution
Ceratocystis coerulescens	Sugar maple	Sap streak	Northern USA
Ceratocystis fagacearum	Red oak	Oak wilt	USA, Europe
Ceratocystis fimbriata	Coffee	Trunk rot	S. America
	Fig	Canker	Japan
	Mango	Wilt	Brazil
	Poplar	Canker	N. America, Europe
	Rubber	Mouldy rot	Brazil
	Sweet potato	Black rot	E. Asia
	Sycamore	Canker	Europe
Ceratocystis lariciola	Larch	Canker stain	Europe
Ceratocystis paradoxa	Citrus	Soft rot	India
	Coconut	Stem bleeding	Asia
	Pineapple	Pineapple disease	Tropical
	Sugar cane	Pineapple disease	Tropical
Leptographium wageneri	Conifers	Black stain	N. America
Ophiostoma minus	Pines	Canker stain	N. America
Ophiostoma montia	Pines	Canker stain	N. America
Ophiostoma polonica	Spruce	Canker stain	Europe
Ophiostoma ulmi/novo-ulmi	Elms	Dutch Elm Disease	N. America, Europe, Asia, New Zealand
Sporothrix schenkii	Humans	Sporotrichosis	Cosmopolitan

1.4.3.3 Taxonomy of sapstain fungi

Different concepts have been used to define fungal species. The morphological concept is the classical approach, where units are defined on the basis of morphological features. The biological concept uses classical genetics and emphasises gene exchange within species. This concept can only be applied to sexual fungi. Modern methods including molecular species-specific antibodies, DNA probes, physiological and biochemical tests, secondary metabolites, fatty acid composition, have now been used in classification and identification of species (Guarro *et al*, 1999).

1.4.3.3.1 Taxonomy of *Sphaeropsis sapinea*

The nomenclature of *S. sapinea* has been subject to considerable confusion. The fungus over the last 150 years acquired at least 23 synonyms – *Diplodia pinea* was the most popular (Swart *et al*, 1993). *S. sapinea* is an anamorphic Ascomycete. Its sexual state is believed to be *Botryosphaeria* that belongs to the Loculoascomyctes, family *Botryosphaeriaceae.*

Species of *Botryosphaeria* are distributed in temperate and tropical climates worldwide. The genus produces anamorphs placed in the form-genera *Fusicoccum*, *Dothiorella*, *Diplodia*, *Lasiodiplodia*, *Sphaeropsis* and *Phyllosticta* (Jacobs and Rehner, 1998). The teleomorph of *Botryosphaeria* is rarely encountered in nature, therefore the taxonomy of the group is based primarily on characteristics of the anamorphs (Jacobs and Rehner, 1998). Species identification in *Botryosphaeria* is complicated because the taxonomy of the anamorphs is confusing and some morphological characters believed to be informative at the species level including conidial pigmentation, septation and stromata morphology show extensive plasticity (Jacobs and Rehner, 1998). Fruiting structures of two or three *Botryosphaeria* species were found together on a single host (Jacobs and Rehner, 1998). Thus, identification of the *Botryosphaeria* species using teleomorph or anamorph structures is not reliable for definitive species identification.

Botryosphaeria species form microscopic flask shaped fruiting bodies called pycnidia (Figure 1.8). The inside layer of the pycnidial wall is covered with conidiophores or conidiogenous cells releasing conidia into the centrum of the pycnidium which eventually ooze out through the neck (Eaton and Hale, 1993).

More specific ecological information pertaining to *S. sapinea* especially in New Zealand is provided in Section 1.4.4.1.

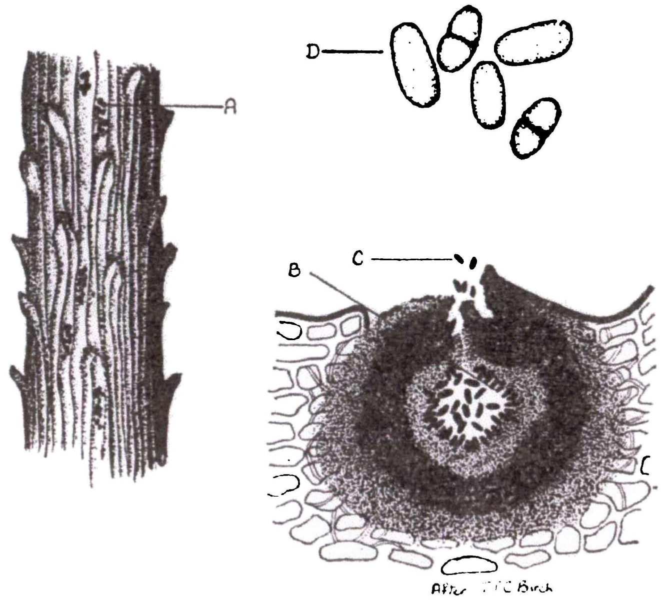


Figure 1.8: *Sphaeropsis sapinea:* A and B, pycnidium on needle and in longitudinal section; C and D, conidia (source Lancaster, 1955).

1.4.3.3.2 Taxonomy of the Ophiostomataceae Family

The taxonomy of the sapstain fungi belonging to the *Ophiostoma* species has been subjected to many changes. An overview of the classification of the genus *Ophiostoma* (including the asexual states) within the division Ascomycota (Kingdom: Fungi) is given in Figure 1.9. *Ophiostoma* species belong to the class Pyrenomycetes, and the order Ophiostomatales and the genus *Ophiostoma*, which comprise more than a hundred species. The Ophiostomataceae family represents an artificial grouping of morphologically similar genera, including *Ophiostoma*, *Ceratocystis* and *Ceratocystiopsis*.

The term *Ceratocystis sensu lato* is commonly used to address the three genera *Ophiostoma*, *Ceratocystis sensu stricto*, and *Ceratocystiopsis*. However, recent comparison of ribosomal DNA sequences indicates that *Ophiostoma* and *Ceratocystiopsis* are most likely synonyms, while *Ophiostoma* and *Ceratocystis sensu stricto* are only distantly related. Excellent reviews of the taxonomic history of this group of fungi are found in De Hoog (1974), De Hoog and Scheffer (1984) and Upadhyay (1993).

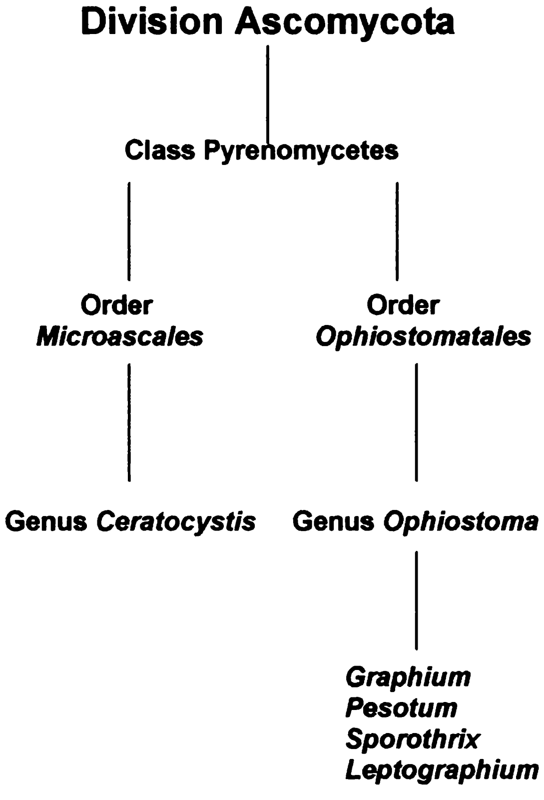


Figure 1.9: The classification of the genera *Ophiostoma*, including the *Ophiostoma* anamorphs *Graphium*, *Pesotum*, *Leptographium* and *Sporothrix* within the division Ascomycota.

Although morphologically similar, the members of the Ophiostomataceae family differ in cell wall composition, anamorphs and cycloheximide sensitivity. Species of *Ophiostoma* and *Ceratocystiopsis* form conidia through apical wall building (Minter *et al*, 1983; Harrington, 1987). These species also contain cellulose, chitin and rhamnose in their cell walls (Smith *et al*, 1967; Spencer and Gorin, 1971; Jewell, 1974) and are tolerant to high concentrations of cycloheximide (Harrington, 1981). *Ceratocystiopsis* species are ecologically and taxonomically related to *Ophiostoma* and are separated from the latter primarily on the basis of their elongated ascospores with falcate sheaths (De Hoog and Scheffer, 1984).

Ceratocystis species form conidia through a ring wall building process and are characterised by the anamorph *Chalara* (Benade *et al*, 1995). The cell walls of *Ceratocystis* species are composed primarily of chitin with no detectable cellulose or rhamnose and like most eukaryotes, they are sensitive to low concentrations of cycloheximide. Table 1.4 shows a summary of the major differences between *Ceratocystis* species and *Ophiostoma* species.

Table 1.4: Non-molecular characteristics used to separate *Ceratocystis* species and *Ophiostoma* species.

***Ceratocystis* species**	***Ophiostoma* species**
Enteroblastic anamorph	**Holoblastic anamorph**
(De Hoog, 1974)	(De Hoog, 1974)
Cycloheximide sensitive	**Cycloheximide resistance**
(Harrington, 1981)	(Harrington, 1981)
Cellulose absent in the cell wall	**Cellulose present in the cell wall**
(Smith *et al*, 1967; Jewell, 1974)	(Smith *et al*, 1967; Jewell, 1974)
Rhamnose absent in cell wall	**Rhamnose present in cell wall**
(Spencer and Gorin, 1971; Weijman and De Hoog, 1975)	(Spencer and Gorin, 1971; Weijman and De Hoog, 1975)
Young asci line periphery of inner perithecium	**Young asci produced from base of inner perithecium**
(van Wyk *et al*, 1993)	(van Wyk *et al*, 1993)

Through the development of molecular information on the Ophiostomataceae family it has been possible to determine that *Ceratocystis* and *Ophiostoma* are phylogenetically distinct (Spatafora and Blackwell, 1994). Based on partial sequences of the small subunit ribosomal RNA it appears that *Ceratocystis* is phylogenetically best accommodated in the Microascales and *Ophiostoma* in Diaporthiales (Berbee and Taylor, 1992; Spatafora and Blackwell, 1994).

Viljoen *et al* (2000) attempted to use computer-aided systematics to evaluate the morphological characters of the Ophiostomataceae family. Analysis of equally weighted morphological characters of the Ophiostomataceae family produced tree topologies similar to those from studies using molecular analysis. In addition, the data showed that morphologically *Ceratocystis* species forms a distinct monophyletic group to *Ophiostoma* and *Ceratocystiopsis*. Anamorph characters were important in distinguishing the different groups.

1.4.3.3.3 Anamorphs

Mycologists have long used a system of classification that allows anamorphs to be named separately from the holomorph of which they form a part. As a consequence many fungi can have two different names. Identification of species in anamorph genera remains a difficult problem. Without a sexual state, mating compatibility among strains cannot be determined therefore reliance is on acceptable morphological variation and molecular techniques to distinguish between species. Members of the Ophiostomataceae family have anamorphs in a number of genera some of which are shown in Figure 1.10. The taxonomy of these states is confused and controversial (Upadhyay and Kendrick, 1975; Upadhyay, 1993). However, the anamorphs have directed the taxonomy of this group of fungi (Samuels, 1993). The genus *Ophiostoma* is mainly associated with the anamorph genera *Graphium, Pesotum, Leptographium, Sporothrix, Hyalorchinocladiella*, and *Hyalodendron* (Upadhyay, 1993). The genera *Sporothrix* and *Graphium* commonly occur in the same species of *Ophiostoma*. Besides the ascospores (sexual spores), *Ophiostoma* species produce three different anamorph spore types: *Graphium (Pesotum)*-type spores, *Sporothrix (Cephalosporium)*-type spores, and yeast-like spores. In contrast, the *Ceratocystis* species produce one distinct anamorph, *Chalara* (Upadhyay, 1993).

Figure 1.10: Structures common to *Ophiostoma* and *Ceratocystis* and their anamorphs (source Käärik, 1980; Wolfaardt *et al*, 1992).

1.4.3.3.4 *Leptographium* species.

The anamorph genus *Leptographium* includes both forest pathogens and species commonly associated with sapstain of logs and lumber. Most species of *Leptographium* are closely associated with insects, particularly bark beetles (Coleptera: Scolytidae) that act as their vectors. The best-known plant pathogen is the black-stain root disease caused by three varieties of *Leptographium wageneri* (Harrington and Cobb, 1987; Cobb, 1988). Some *Leptographium* species are known anamorphs of *Ophiostoma* (Harrington, 1987).

As in the case of *Ophiostoma*, *Leptographium* species are able to tolerate high concentrations of cycloheximide (McCall and Merrill, 1980; Harrington, 1981).

Leptographium species are characterised by a darkly pigmented very wide single hyphal stalk and which branches profusely at the apex (Kendrick, 1962). In *L. procerum*, a conidiogenous apparatus of three to five series of branches terminates in the conidiogenous that produce obovoid conidia with truncate ends (Kendrick, 1962)

The genus *Leptographium* has been associated with several different but morphologically similar genera. *Verticicladiella* and *Phialocepaia*, two genera described in the *Leptographium* complex, were distinguished from *Leptographium* based on differences in conidium development (Hughes, 1953; Kendrick, 1961; Kendrick, 1962). Subsequently, a detailed scanning electron microscopic examination of conidial development in *Leptographium* and *Verticicladiella* revealed that various species in these genera develop both sympodially and percurrently (Wingfield, 1985), thus *Verticicladiella* was reduced to synonymy with *Leptographium*.

1.4.3.3.5 *Sporothrix* species

In 1909, Hektoen and Perkins established the genus *Sporothrix* for the agent of human sporotrichosis, *S. schenckii* (De Hoog, 1993). Sporothrix species are characterised by transparent cell walls, dehydrogenated coenzyme Q-10 systems and simple, central septal pores (Suzuki and Nakase, 1986; De Hoog, 1993). They also produce elongated conidiogenous cells that arise from undifferentiated hyphae. These cells terminate in clusters of conidium-bearing denticles that extend sympodially and carry hyaline conidia (De Hoog, 1974). Several *Sporothrix* species have rhamnose in their cell wall, a characteristic they share with the *Ophiostoma* species (De Hoog, 1993).

1.4.3.3.6 *Graphium/Pesotum* species

Originally synnematous anamorphs of *Ophiostoma* species were placed in the genus *Graphium*, though it is now thought that *Graphium* species are anamorphs of Microascales (Okada *et al*, 1998). The genus name *Pesotum* is available for the anamorphs of *Ophiostoma* (Harrington *et al*, 2001). *Pesotum* was based on the anamorph of *O. ulmi* and was characterised by the synnematous anamorph as well as a *Sporothrix* (Crane and Schoknecht, 1973). The genus *Pesotum* was used for all synnema forming species with affinities to *Ophiostoma*, even those with no *Sporothrix* anamorph (Okada *et al*, 1998).

1.4.4 New Zealand sapstain fungi

Yeates (1924) after investigating sapstain of *Podocarpus dacrydioides* (white pine) for the New Zealand State Forest Service also described sapstain as a problem on *P. radiata*. Yeates found perithecia associated with sapstain in *P. radiata* from one site in Taranaki, North Island, New Zealand. The species found were predominately members of the Ophiostomataceae family (Yeates, 1924). Curtis (1926) first recorded *S. sapinea* in New Zealand, from diseased shelterbelts in the Marlborough and Nelson districts. In 1932, the State Forest Service was faced with a late winter and early spring epidemic of disease covering large areas of its exotic forests of *P. radiata* (Birch, 1936) associated with *S. sapinea*. From these accounts it is unsure when *S. sapinea* was first introduced into New Zealand but it was widespread throughout the country by 1936 (Birch, 1936).

In the late 1960's to 1980's, two groups examined the causative agents of sapstain in New Zealand (Butcher, 1968a; Hutchison and Reid, 1988a,b). Butcher (1968a) studied the occurrence, distribution and development of sapstain and decay on posts of *P. radiata* at two sites in the central North Island (Tahorakuri and Waipa). Hutchison and Reid (1988a, b) sampled both native and exotic tree species from a few areas of the North Island including Kaingaroa Forest and within the Auckland and Coromandel areas. The organisms identified were as follows: *Sphaeropsis sapinea*, *Sphaeronaemella fimicola*, *Hyalopesotum pini*, *Ceratocystiopsis falcata*, *Ceratocystis piceaperdem*, *C. ips*, *C. novo-zealandiae*, *C. piceae*, *C. coronata*, *C. rostrocoronata*, *C. pilifera* and *Leptographium* species (Hutchison and Reid, 1988a,b). These studies did not link organisms with their overall New Zealand distribution nor their contribution to sapstain in *P. radiata* and other New Zealand wood species.

Recently, a nationwide survey of New Zealand sapstain fungi identified the presence of *S. sapinea* and members of the Ophiostomataceae genera (Farrell *et al*, 1997; Farrell *et al*, 1998, Kay *et al*, 1998). The members of the Ophiostomataceae included *Ophiostoma*, *Leptographium*, *Pesotum* and *Sporothrix*. The sapstain species isolated and identified from New Zealand during the first 15 months of the survey (September 1996 to November 1997) are listed in Table 1.5 along with the known geographic range other than New Zealand.

Table 1.5: *Ophiostoma* species present in New Zealand and their known world distribution (adapted from Kay *et al*, 1998).

***Ophiostoma* species present in New Zealand**	**Known geographic range other than New Zealand**
Leptographium procerum	North America, Europe, Asia, New Zealand (Wingfield *et al*, 1988)
Leptographium truncatum	North America, Europe, Asia, (Kaneko and Harrington, 1990) Africa, New Zealand (Wingfield and Marasas, 1983)
Ophiostoma coronata	North America (Olchowecki and Reid, 1974), New Zealand (Hutchison and Reid, 1988a)
*Ophiostoma floccosum**	North America, Europe, New Zealand, Australia, Asia (Harrington *et al*, 2001, Uzunovic *et al*, 2000), Chile
*Ophiostoma galeiformis**	Europe (Bakshi, 1951)
*Ophiostoma huntii**	North America (Robinson-Jeffrey and Grinchenko, 1964), Europe (Jacobs and Wingfield, 2001)
Ophiostoma ips	North America, Europe, Asia (Hunt, 1956), Africa (Wingfield and Marasas, 1980), New Zealand (Hutchison and Reid, 1988a)
*Ophiostoma nigrocarpum**	North America (Raffa and Smalley, 1988a,b)
Ophiostoma piceae	Europe, North America (Harrington *et al*, 2001), New Zealand (Hutchison and Reid, 1988a), Chile (Butin and Aquilar, 1984)
Ophiostoma piliferum	United States, Europe, (Hunt, 1956), Canada (Olchowecki and Reid, 1974), South America (Butin and Aquilar, 1984)
*Ophiostoma pluriannulatum**	North America, Europe (Hunt, 1956)
*Ophiostoma querci**	North America, Europe, Africa, Asia, Australia (Harrington *et al*, 2001)
*Ophiostoma setosum**	North America (Uzunovic *et al*, 2000), New Zealand, Australia, Chile (Harrington *et al*, 2001)
*Ophiostoma stenocerus**	North America (Griffen, 1968), Europe (Robak, 1932)
Sporothrix sp. A	unknown
Sporothrix sp. B	unknown
Sporothrix sp. C	unknown

* First reported in New Zealand.

1.4.4.1 *Sphaeropsis sapinea* in New Zealand

S. sapinea is an important opportunistic pathogen of *P. radiata* and other conifer species and is associated with significant damage in exotic plantations in New Zealand, Australia and South Africa. The majority of research on *S. sapinea* was dedicated to latent infections caused by this fungus in living trees. In standing *P. radiata*, *S. sapinea* causes bud-wilt, whorl cankers, stem depressions, shoot/leader dieback and crown wilt (Birch, 1936; Marks and Minko, 1969; Chou, 1976a, 1987).

S. sapinea also grows as a saprophyte in dead bark, wood, needles, cones and general forest debris (Birch, 1936). Post-harvest infections of *S. sapinea* result in the formation of visual sapstain (Birch 1936; Butcher 1967; Butcher 1968a). The widespread distribution of *S. sapinea* in *P. radiata* plantations was attributed to prolific spore-production and subsequent dissemination by means of wind and rain.

Until recently, only two morphotypes, A and B, were described for *S. sapinea* both distinguishable based on the difference in cultural and conidial characteristics, as well as pathogenicity. Type A isolates were described as having fluffy white to grey-green mycelia, smooth-walled conidia, lacking in microconidia, and as being pathogenic to both wounded and unwounded hosts (Swart *et al*, 1991; Swart *et al*, 1993). In contrast, type B isolates were described as having white to black closely appressed mycelia, pitted conidia, producing microconidia and requiring a wound for infection (Swart *et al*, 1991; Swart *et al*, 1993). The distinction between A and B morphotypes were confirmed using random amplified polymorphic DNA (RAPD) markers (Smith and Stanosz, 1995; Stanosz *et al*, 1996). Three morphotypes are now described for *S. sapinea* (de Wet *et al*, 2002). A third group (C) was identified using RAPD markers in isolates from Indonesia and Mexico (de Wet *et al*, 2000). Conidia of this group were significantly longer than those of both A and B. Isolates of the C group were also found to be more virulent on seedlings than the other two groups (de Wet *et al*, 2002). Birch (1936) speculated that within New Zealand there was a lack of variability within the *S. sapinea* population indicating the presence of only a single strain. Subsequent reports showed uniformity among the New Zealand isolates and conformity to the description of the A morphotype (Chou, 1976b; Swart *et al*, 1993). Recent examination of the growth, cultural and conidial morphology, carbon and nitrogen utilisation and pathogenicity of *S. sapinea* found significant differences among New Zealand isolates (Kay *et al*, 2002). This result differed from the previous reports of Chou (1976b). Given the asexual nature of *S. sapinea* reproduction, the differences between the level of morphological and cultural variability observed suggested a re-introduction of *S. sapinea*, the spread of additional isolates or a shift in the population (Kay *et al*, 2002). The morphological and cultural features of several of the New Zealand isolates were consistent with the known B and C types (Kay *et al*, 2002). The presence of a species closely related to *S. sapinea*, *Botryosphaeria* species was isolated from *P. radiata* standing trees in New Zealand (Harrington, personal communication).

1.4.4.2 *Ophiostoma* species within the *Ophiostoma piceae* complex

The *O. piceae* complex is a monophyletic group of insect-dispersed ascomycetes with a *Pesotum* anamorph and a *Sporothrix* anamorph that include *O. piceae*, *O. setosum*, *O. querci* and *O. floccosum* isolated from New Zealand (Harrington *et al*, 2001). The nine recognised species within the *O. piceae* complex are delimited by synnema morphology, growth rate at 32°C, mating reactions and the sequences of the internal transcribed spacer (ITS) region of the rDNA operon (Harrington *et al*, 2001).

O. piceae was the oldest described species of this complex of morphologically similar species and as a result much of the older literature (pre 1990) reporting on *O. piceae* is firstly extensive and secondly suspect with regard to current species identification (Harrington *et al*, 2001). The majority of the literature described *O. piceae* as a common sapstain fungus found mainly on Pinaeceae hosts throughout Europe, North America and Japan (Brasier and Kirk, 1993; Halmschlager *et al*, 1994; Pipe *et al*, 1995; Kim *et al*, 1999). In New Zealand, Butcher (1968b) recorded *O. piceae* on red beech (*Nothofagus fusca*) and silver beech (*Nothofagus menziesii*) and also described it as a minor sapstain species on *P. radiata.* The teleomorph and anamorphs of this species lack distinctive features, which led to the proposal of numerous synonyms, all of which are now described as distinct species (Harrington *et al*, 2001). Work done in past literature on *O. piceae* as a sapstain fungus was difficult to interpret with the knowledge that misidentification of the *O. piceae* complex was possible.

O. querci was first isolated from declining oaks in Europe and was treated by Hunt (1956) as a synonym of *O. piceae*. Pipe *et al* (1995) described two distinct populations within *O. piceae*, OPH and OPC, the former associated with oak decline and the latter isolated from conifers. No clear difference in conidial size of the anamorph states could be obtained (Halmschlager *et al*, 1994). Pryzbyl and Morelet (1993) studied morphological differences between *O. piceae* and *O. querci*, and found that *O. querci* differs from *O. piceae* in lengths of synnematal conidiogenous cells, perithecial necks, and ostiolar hyphae. Other physiological and biochemical differences, as well as, a strong reproductive isolation between OPH and OPC were also found (Brasier and Stephens, 1993; Halmschlager *et al*, 1994; Pipe *et al*, 1995). Molecular analysis using RAPD markers could clearly distinguish unique bands for OPH and OPC (Halmschlager *et al*, 1994; Pipe *et al*, 1995). It was recommended that the name *O. querci* be reinstated for the OPH strains (Pipe *et al*, 1995). *O. querci* appeared to be a widespread species and was commonly found on conifers and hardwoods throughout Europe and North America (Brasier and Kirk, 1993; Halmschlager *et al*, 1994; Pipe *et al*, 1995; Kim *et al*, 1999). *O. querci* was isolated from *P. radiata* in New Zealand along with *O. piceae* (Farrell *et al*, 1998).

O. floccosum was originally isolated from *Pinus* species in Sweden (Mathiesen-Käärik, 1960) Many studies described *O. floccosum* as being a synonym of *O. piceae* (Griffen, 1968; Przybyl and de Hoog, 1989), until recently when Harrington *et al* (2001) described it as a distinct species. *O. floccosum* was first described in New Zealand as a *Graphium* species easily identified by its characteristic red brown synnema (Farrell *et al*, 1998). The ITS sequence data for *O. floccosum* was similar to *O. piceae* (Harrington *et al*, 2001).

O. setosum and its anamorph *Pesotum cupulatum* were only recently described by Uzunovic *et al* (2000) and Harrington *et al* (2001), respectively. This species is a member of the *O. piceae* complex and can most easily be distinguished morphologically from the other species of the complex by the dark, seta-like marginal hyphae on the outside of the synnematous structure (Harrington *et al*, 2001). This species prefers cooler temperatures for growth and perithecia production, and is probably native to the Pacific Northwest of North America. This fungus was isolated from *P. radiata* in New Zealand and California (Farrell *et al*, 1998; Harrington *et al*, 2001) as well as *Tsuga* species and *Psuedotsuga menziesii* from Washington, USA (Harrington *et al*, 2001). Uzunovic *et al* (2000) identified isolates from *Picea*, *Pinus* and *Tsuga* species from British Columbia, Canada and Oregon, USA.

1.4.4.3 *Ophiostoma* species with only *Sporothrix* anamorphs

This group of fungi are characterised by mycelium composed of *Sporothrix* conidiophores including the New Zealand isolates of *O. piliferum*, *O. pluriannulatum* and *O. stenocerus*. A number of species are also included in this group where only a *Sporothrix* anamorph was found in New Zealand and are named *Sporothrix* species D and X.

In 1878, Hartig assumed that all sapstain in wood particular from Europe was caused by one species, *O. piliferum* (quoted in Seifert, 1993). *O. piliferum*, along with *O. ips*, was one of the most common causes of sapstain in the United States (Seifert, 1993) and in Canada this fungus occurred widely on both coniferous and deciduous tree species (Olchowecki and Reid, 1974). Hutchison and Reid (1988a) believed that the *Ophiostoma* species described by Yeates in 1924, was *O. piliferum*. *O. piliferum* produces superficial perithecia on wood and agar, which develop rapidly in culture. The growth on agar is first white with abundant aerial mycelium becoming brown to black (Griffen, 1968). *O. piliferum* caused a brownish to black stain on all wood types tested by Hutchison and Reid (1988a).

O. pluriannulatum was first described by Hedgcock from the United States on *Quercus* species in 1906 (quoted in Seifert, 1993). Subsequent reports isolated this fungus from

Canada and Europe (Hunt, 1956). The perithecia are distinct as they may produce annulations, which are multiple sets of ostioler hyphae and ascospore masses along the length of the neck due to neck proliferation (Hausner *et al*, 1993).

Griffen (1968) considered *O. stenocerus* not to be an important sapstain fungus, however, there was much speculation that the human pathogen *Sporothrix schenckii* may be the anamorph of *O. stenocerus* (Summerbell *et al*, 1993). This fungus is homothallic and will form abundant perithecia on malt yeast extract agar, with the perithecia forming distinct concentric rings.

1.4.4.4 *Ophiostoma* species with *Leptographium* anamorphs

O. huntii was first described in Canada (Robinson-Jeffrey and Grinchenko, 1964). More recently, *O. huntii* was isolated from many parts of the world (Jacobs *et al*, 1998). *O. huntii* was found to be associated with several bark beetles, including *H. ater* (Harrington, 1988; Jacobs *et al*, 1998). This fungus produces distinct serpentine hyphae, which distinguishes it from other *Leptographium* species.

Like many species of *Ophiostoma*, its anamorph *Leptographium* was shown to cause stain on timber and logs. *L. procerum* and *L. truncatum* were isolated in New Zealand as sapstain organisms (Farrell *et al*, 1998) and from roots of diseased pines (Wingfield and Marasas, 1983; Wingfield and Gibbs, 1991).

L. procerum was isolated in Europe and North America associated with white pine decline on *Pinus strobus* (Kendrick, 1962; McCall and Merrill, 1980; Wingfield *et al*, 1988). In the North Island of New Zealand, *L. procerum* was isolated from black stained sapwood of dead and dying *Pinus strobus* tress in two separate forests (Shaw and Dick, 1980). The presence of *L. procerum* in New Zealand was likely due to its introduction with either the bark beetles *Hylastes ater* or *Hylurgus ligniperda* (Wingfield and Gibbs, 1991). *L. procerum* is characterised by long conidiophores with two or three primary branches on the stipe (Kendrick, 1962). *L. procerum* is also be recognised by its colonies in which conidiophores are arranged to form dark concentric rings on the surface of malt extract agar.

Wingfield and Marasas (1983) described *L. truncatum* from *Pinus* species in South Africa and New Zealand. Strydom *et al* (1997) reduced *L. truncatum* to a synonym with *L.*

lundbergii based on morphological features. Since *L. truncatum* was described from New Zealand (Wingfield and Marasas, 1983) and no sequence comparisons were made between *L. truncatum* and *L. lundbergii*, this thesis will continue with the use of the name *L. truncatum*. It is easily distinguished from *L. procerum* by the absence of concentric rings and aroma on malt extract agar.

1.4.4.5 Other *Ophiostoma* species identified in New Zealand

O. galeiformis was found on larch in Scotland (Bakshi, 1951) and in Sweden (Mathiesen-Käärik, 1960). Reports of this fungus were rare, however, it was always associated with bark beetle galleries. The anamorph exhibits a range of morphologies from a *Leptographium* to a *Pesotum* with the synnema appearing to be a loose aggregation of the *Leptographium* (Baskhi, 1951). Mathiesen-Käärik (1960) described *O. galeiformis* as a secondary stain fungus. This fungus produced a greyish blue discolouration, which was severe on Scots pine but mild on spruce and very light or none at all on larch (Baskhi, 1951).

O. ips was one of the most common species isolated in New Zealand (Farrell *et al*, 1998). Rumbold (1931) first isolated *O. ips* from *Pinus* species and this fungus was subsequently reported in the United States, Japan, Europe (Hunt, 1956), South Africa (Wingfield and Marasas, 1980), Canada (Olchowecki & Reid, 1974) and in New Zealand (Hutchison and Reid, 1988a). *O. ips* was mainly associated with Scolytid bark beetles, and was believed to be introduced into the Southern Hemisphere together with the bark beetle, *Hylastes ater* (Wingfield and Marasas, 1980). There was much confusion over the placement of the anamorphs of *O. ips* (Benade *et al*, 1995). Benade *et al* (1995) believed that the anamorph of this fungus was best considered as a species of *Hyalorhinocladiella*.

O. ips was shown to be a minor pathogen of some tree species. Mathre (1964) studied the pathogenicity of *O. ips* on small *P. ponderosa* trees and believed that the fungus allowed the entry of air into the sapwood causing permanent breakage of the water columns. Klepzig *et al* (1991) suggested that *O. ips* vectored by *Ips pini* caused the death of red pine.

O. ips is easily distinguished by its brown colour, fast growth and ascocarps with rectangular sheath (Hutchison and Reid, 1988a). Griffen (1968) found that *O. ips* produced a severe and extremely blue-black discolouration in conifer sapwood and was almost always associated with bark beetles and other wood inhabiting insects. Hutchison and Reid (1988a) noted that this fungus caused a brownish to black stain in a variety of wood samples.

1.4.5 Ecological aspects of sapstain fungi

Ecology can be defined as the study of organisms in relation to their environment. There are several ways of studying fungal ecology as stated by Dix and Webster (1995). The autecological approach focuses on a given species and attempts to study its behaviour in relation to its changing substratum and to external abiotic and biotic variables. Another approach is to study the fungal communities, which develop on particular substratum. It is also important to define what aspect of the relationship between the fungus and the environment is being studied (Dix and Webster, 1995). Aspects that may be studied include: distribution on a microscopic, macroscopic or geographical scale, presence or absence of a fungus, or relative abundance of a particular species.

Beyond pure collection lists, there were few papers relating to the ecology and distribution of sapstain fungi in forest ecosystems and processing sites. A small survey was undertaken in Canada that identified the sapstain organisms present in a variety of wood species of both timber and logs from different mill locations (Uzunovic *et al*, 1999). The most commonly encountered genus *Ophiostoma* was represented by nine species in this survey and logs had a more diverse range of fungi than lumber. This study also found that no one fungal species occurred exclusively in a particular region or wood substrate (Uzunovic *et al*, 1999).

The role of climate in the population dynamics of sapstain fungi requires more comprehensive investigations. Findley (1959) stated that softwood species were more susceptible to sapstain when harvested in Northern Hemisphere spring or summer than when harvested in winter. According to Findley (1959), it was merely that sapstain fungi do not attack wood during cold weather, and by the time warmer weather arrived, wood that was harvested in winter was partly seasoned and so became less susceptible to attack. In this context, Rogister (1955; quoted in Findley, 1959) carried out a series of experiments on samples taken from Poplar trees harvested in different seasons of the year. Logs harvested in March/April and in July showed the maximum susceptibility to *L. theobromae* whereas logs cut in December, May and June were less infected by sapstain. Trees harvested in autumn became susceptible to sapstain infection in the following

February and March. Presumably, changes in the amount and nature of the reserve materials in the living parenchyma cells of the wood were responsible for these variations in susceptibility. The amount of starch that is stored in the parenchyma cells varies according to season increasing steadily in the summer and decreasing in autumn (Sauter and van Cleve, 1994).

Miller and Goodell (1981) found that severe sapstain developed on *P. ponderosa* stored outdoors during late autumn, early winter in Oregon, U.S.A. Isolations made from the wood identified *O. piliferum* as the cause of the stain. Further studies were conducted and the results indicated that the isolate of *O. piliferum* caused degrading stain in solidly piled pine sapwood lumber stored for one to two months at 3°C to 8°C (Miller and Goodell, 1981).

Much of the ecological information on sapstain fungi worldwide was incidental to taxonomic studies or to investigations of control methods. Other studies on the ecology of sapstain fungi to date are mainly concerned with dissemination of sapstain fungi and their relationships with insects.

1.4.6 Dissemination of sapstain fungi

The dissemination of thousands of spores from one sporing head under optimal conditions is an important factor in the establishment of successful infection and colonisation (Eaton and Hale, 1993). Spores germinate when the environmental conditions, for example, temperature, pH, and moisture levels are suitable for growth and when the nutritional status of the substratum will support fungal growth (Eaton and Hale, 1993).

The sapstain fungi that cause post-harvest deterioration can gain access to the wood in several different ways. The most studied means of dissemination of spores is by insect vectors. Spores are the major agent of infection, but logs in contact with soil or logs stacked in close proximity to each other may be infected by the spread of mycelium (Eaton and Hale, 1993).

1.4.6.1 Wind and rain

It was first thought that air currents played a major role in infection of sapstain on freshly harvested wood (Findley, 1959). The air in the timber yards and mills were thought to contain large numbers of fungal spores, as well as, fragments of mycelium from the dust produced during sawing of infected logs (Björkman, 1946; Mathiesen-Käärik, 1955). Dowding (1969), however, studied various experimental approaches to examine dispersal in dry air, mist and splash droplets of the conidia of *O. piceae*, *O. piliferum*, *O. minus* and *C. coerulescens*. It was found that both mist laden air and splash droplets would dislodge and disseminate spores easily, whereas, dry air was completely ineffective in causing conidium dispersal.

Gregory *et al* (1959) showed that raindrops falling at terminal velocity and drops falling more slowly from vegetation could disperse fungal spores. Splash from rain could act as a complete dispersal mechanism in still air or as a "take off" mechanism leading to dispersal of fungal spores by wind.

The biology and ecology of *S. sapinea* is different from that of the *Ophiostoma* species. Pycnidia of *S. sapinea* develop on dead pine needles and forest litter (Swart and Wingfield, 1987). Conidia are released in the presence of moisture and are disseminated by rain splash and wind (Brookhouser and Peterson, 1971; Swart and Wingfield, 1987). Feci *et al* (2002) studied the relationship between *S. sapinea* and the cone bug *Gastrodes grossipes* in *Pinus nigra* in Italy. They found a high frequency of insects carrying conidia of *S. sapinea* and implied a role for *G. grossipes* as a disseminating agent of this fungus.

1.4.6.2 Insect dispersal and interactions

Bark beetles play an important role in disseminating spores of *Ophiostoma* in standing trees, as well as, in logs and timber. The relationship between fungi and insects however, remains uncertain. Clearly the fungus benefits by being dispersed and the fruiting structures of the *Ophiostoma* are obvious adaptations for insect dispersal (Harrington, 1993). However, benefits to the beetle are less clear (Harrington, 1993). Most authors support the notion that beetles and fungi are mutualistic symbionts (Harrington, 1993). Both fungi and bark beetles can exist and develop normally without each other but they are almost always found together in nature (Griffen, 1968).

Ophiostoma species are well adapted for insect dispersal, possessing evanescent asci and spores with sticky sheaths that collect in gelatinous masses at the apex of perithecial necks. The spores of *Ophiostoma* are most commonly dispersed as they adhere to the exoskelton of bark beetles or as they are eaten and passed through the digestive tract (Harrington, 1993). Some beetles have special fungus carrying structures, mycangia, which are typically simple pits or pockets in the exoskelton (Livingston and Berryman, 1972; Levieux *et al*, 1989).

Two types of exotic bark beetles, *Hylastes ater* and *Hylurgus ligniperda*, which are native to Europe, occur on seedlings and stumps of mature *P. radiata* in New Zealand. They undergo maturation feeding on healthy pine seedlings and thus can cause serious damage (Reay and Walsh, 2001). Reay *et al* (2002) undertook the first comprehensive survey of fungi associated with the bark beetles, *H. ater* and *H. ligniperda*, in New Zealand. *H. ater* was shown conclusively to be a vector of sapstain fungi in second rotation *P. radiata* forests (Reay *et al*, 2002). Table 1.6 shows the sapstain fungi isolated from *H. ater* and from seedlings following attack by *H. ater*. *O. huntii* and *Leptographium* species were thought to have become established in New Zealand due to their association with *H. ater* or *H. ligniperda* (Wingfield and Gibbs, 1991; Jacobs *et al*, 1998).

Table 1.6: Sapstain fungi isolated from *Hylastes ater* and seedling following attack of *Hylastes ater* in New Zealand (source Reay *et al*, 2001).

Sapstain fungi isolated from *H. ater*	**Sapstain fungi isolated from seedlings following attack by *H. ater***
L. procerum	*L. procerum*
L. truncatum	*L. truncatum*
O. galeiformis	*O. floccosum*
O. huntii	*O. galeiformis*
O. ips	*O. huntii*
O. pluriannulatum	*O. piceae*
O. querci	*O. querci*
O. setosum	*O. setosum*

Bark beetles have been the most studied insect vectors of sapstain fungi, but the casual association between other wood inhabiting insects has also been reported. Fungus feeding and predatory diptera, beetles, mites and other small animals are active in bark beetle galleries and may also be important vectors of *Ophiostoma* species (Bridges and Moser, 1983; Moser, 1985). Mites and other microfauna frequently feed upon *Ophiostoma* species and may ensure dissemination through this activity (Leach *et al*, 1934).

2 General materials and methods

2.1 Fungal isolates

All sapstain fungi used and reported in this thesis were collected from either the survey of New Zealand sapstain fungi conducted by the University of Waikato, the export trials or the biological control trials as part of this thesis research. Table 2.1 gives the strain number, origin, site of isolation in New Zealand and the date of isolation of the sapstain species used in this thesis.

Table 2.1: Details of isolates of sapstain fungi used in thesis.

Species	Strain number*	Origin	Site in New Zealand	Date isolated
Altemaria altemata	11 (S)	General Debris	Warkworth, North Island	25/9/96
L. procerum	417 (S)	Felled log	Riverhead Forest, North Island	6/05/97
L. procerum	446 (S)	Felled log	Mahurangi Forest, North Island	22/05/97
L. procerum	1852 (S)	Felled log	Whitford Forest, North Island	29/01/98
L. truncatum	J404 (O)	*Hylurgus* sp. (beetle)	Kinleith Forest, North Island	15/3/01
L. truncatum	J696 (O)	Stump	Kinleith Forest, North Island	15/3/01
O. coronata	431 (S)	Felled log	Mahurangi Forest, North Island	28/05/97
O. coronata	868 (S)	Felled log	Mahurangi Forest, North Island	20/06/97
O. floccosum	68 (S)	Felled log	Kinleith Forest, North Island	21/10/96
O. floccosum	148 (S)	General sampling	Hanmer Springs, South Island	23/01/97
O. floccosum	J2004 (ET)	Felled log	Kinleith Forest, North Island	31/08/01
O. galeiformis	413 (S)	Felled log	Riverhead Forest, North Island	5/05/97
O. galeiformis	832 (S)	Felled log	Whitford Forest, North Island	9/07/97
O. huntii	474 (S)	Felled log	Tauranga, North Island	15/05/97
O. huntii	903 (S)	*Pinus taeda*, felled log	Tarawera Forest, North Island	26/06/97
O. ips	424 (S)	Felled log	Northland, North Island	22/05/97
O. ips	1024 (S)	Felled log	Mahurangi Forest, North Island	1/08/97
O. ips	P36 (S)	Felled log	Kinleith Forest, North Island	12/01/00
O. nigrocarpum	929 (S)	*Pinus taeda* felled log	Tarawera Forest, North Island	26/06/97
O. piceae	144 (S)	Needles	Hanmer Springs, South Island	6/03/97
O. piceae	174 (S)	Timber	Greymouth, South Island	18/12/97
O. piceae	1566 (S)	Felled log	Kaingaroa Forest, North Island	7/08/97
O. pluriannulatum	151 (S)	General sampling	Abel Tasman National Park, South Island	30/01/97
O. pluriannulatum	847 (S)	*Pinus taeda* felled log	Tarawera Forest, North Island	26/06/97
O. pluriannulatum	962 (S)	*Eucalyptus* sp. log	Northland, North Island	19/06/97
O. querci	162 (S)	*Eucalyptus* sp. chip	Kawerau, North Island	13/02/97
O. querci	1688 (S)	Felled log	Whitford Forest, North Island	30/01/98
O. setosum	670 (S)	Felled log	Tauranga, North Island	14/05/97
O. setosum	J1785 (BC)	Felled log	Kinleith Forest, North Island	9/05/01
O. stenocerus	930 (S)	Branch	Northland, North Island	1/07/97
S. sapinea	D4 (S)	Cone	Dome Forest, North Island	26/09/96
S. sapinea	D33 (S)	Knot, felled log	Tauranga, North Island	8/12/96
S. sapinea	D35 (S)	Live tree	Kinleith Forest, North Island	13/12/96

*the letter in parenthesis represents source of sample – S = Survey, O = other, ET = Export Trial, BC = Biological Control Trial.

In addition three cultures of common wood moulds were obtained from the Horticultural and Food Research Institute of New Zealand Ltd. (Hort Research), Ruakura Research Centre, Hamilton, New Zealand. They included *Epicoccum nigrum* (E125), *Cladosporium cladosporioides* (no strain number) and *Trichoderma koningi* (MTM). A number of albino strains were also used in experimental work in this thesis research and these strains will be described in Chapter 6.

2.2 Media

The follow media were used in this thesis:

Malt extract agar (1.5% unless otherwise stated) – 1.5% malt extract (Becton Dickinson and Company, USA), and 2.0% agar (Germantown, New Zealand) in distilled water.

Malt extract broth- 1.5% malt extract (Becton Dickinson and Company, USA) in distilled water.

Malt yeast extract agar – 0.2% yeast extract (Becton Dickinson and Company, USA), 1.5% malt extract, and 2.0% agar in distilled water.

Media 4 – 0.2% yeast extract, 1.5% malt extract, 2.0% agar, 200mg/L chloramphenicol (Applichem, Germany) and 100mg/L streptomycin sulphate (Applichem, Darmstadt) in distilled water.

Media 6 - 0.2% yeast extract, 1.5% malt extract, 2.0% agar, 200mg/L chloramphenicol, 100mg/L streptomycin sulphate and 400mg/L of the antibiotic cycloheximide (Sigma, USA) in distilled water.

All media were sterilised by autoclaving for 20 minutes at 121°C. The antibiotic chloramphenicol was added prior to autoclaving, but streptomycin sulphate and cycloheximide were filter sterilised after the medium was autoclaved and cooled to 50°C.

2.3 Collection of sapstain fungi

Sapstain fungi were isolated from field environments as described in Chapter 3, 4, 5, and 6. Fungi were collected from a variety of sources including forest debris, logs, timber, wood chips and seedlings. Samples were collected and placed in clean resealable plastic bags and processed as soon as possible to reduce colonisation by aggressive, secondary fungi. Indirect isolations were made after surface sterilization, by soaking specimens in 5% hypochlorite for one minute and rinsing twice in sterile water at room temperature, slivers were then taken aseptically with a sterile scalpel. Slivers were placed on two selective media (Media 4 and Media 6) both media contain malt yeast extract agar but supplemented with antibiotics. Media 4 was supplemented with chloramphenicol to provide as broad-spectrum antibiotic and streptomycin sulphate as antibiotic effective against many gram negative and gram positive bacteria (Seifert *et al*, 1993). Media 4 allowed for the growth of all sapstain fungi as well as many other fungi. Media 6 was identical to Media 4 but contained the antibiotic cycloheximide. *Ophiostoma* species are cycloheximide resistant (Harrington, 1981) unlike *S. sapinea* and many other wood inhabiting or contaminating fungi.

The plates were incubated in a darkened growth chamber at 25°C for between 4 and 21 days. Any resulting cultures were aseptically transferred onto fresh plates of malt extract agar or onto Media 4 or Media 6 if the original plate contained masses of other contaminating fungi.

Spores of the *Ophiostoma* species were collected under a dissecting microscope (Nikon SM 7800) from either the resulting perithecia or synnema using a sterilized needle and transferred to a fresh plate. As *Ophiostoma* species grow in close proximity, isolations were made from single fruiting structures to ensure homogeneity (Seifert *et al*, 1993). Cultures suspected of being sapstain fungi but not producing fruiting structures were transferred from the agar containing the mycelial outgrowth, with a sterile scalpel onto fresh media. Plates were incubated at 25°C and clean subcultures were prepared for identification (Section 2.4) and storage (Section 2.6).

2.4 Identification of sapstain fungi

Pure cultures of sapstain fungi were obtained from field samples as described in Section 2.3. The cultures were identified using morphological features, however if the morphological characteristics of an unknown sapstain fungi were different to species previously seen, mating capabilities and molecular analysis was performed. Unknown *Ophiostoma* species were sent to Doug McNew and Tom Harrington at Iowa State University for molecular analysis as part of FRST UOW 803 programme.

2.4.1 Identification of *Sphaeropsis sapinea*

Cultures suspected of being *S. sapinea* were incubated with *P. radiata* needles. Fresh green needles were collected from a 10 year old *P. radiata* tree and autoclaved prior to use. The needles were placed in a 90mm Petri dish containing the suspected culture of *S. sapinea* and the plate was maintained under ultraviolet light at ambient temperature for up to 14 days. Sterile water (5mL) was placed on the plate containing the needles, after ten minutes the needles were examined microscopically for the presence of pycnidia and subsequent spore release.

2.4.2 Identification of *Ophiostoma* species

Cultures of Ophiostomataceae were identified on the basis of morphological and physiological characters into putative species. Molecular characterisations, particularly DNA sequences of the internal transcribed spacer regions of ribosomal DNA (Harrington *et al*, 2001) and mating capabilities with tester strains were used to confirm unknown *Ophiostoma* species identifications.

2.4.2.1 Morphological Identification

Morphological and physiological characters (Table 2.2, Table 2.3 and Table 2.4) were determined from cultures grown on malt extract agar at room temperature for between 7 and 14 days.

Table 2.2: Distinguishing characteristics for *Ophiostoma* species with only *Sporothrix* anamorphs.

Species	Perithecia with annulations	Ramo-conidia	Colour of protoperithecia	Mycelium with concentric rings[a]	Culture aroma[b]
O. piliferum	No	Common	Grey to black	No	Mushroom
O. pluriannulatum	Yes	Common	Light brown to grey	Rare	No
O. coronata	No	Common	Light brown to grey	Most isolates	No
O. stenocerus	No	Rare	Not seen	Yes	No

[a] Concentric rings of aerial mycelium, microconidiophores and conidia on malt extract agar
[b] Smell on malt extract agar.

Table 2.3: Distinguishing characteristics for *Ophiostoma* species with only *Leptographium* or *Pesotum* anamorphs.

Species	***Leptographium* only**	***Pesotum* only**	***Leptographium* to *Pesotum***	**Serpentine hyphae**	**Perithecia in culture**	**Mycelium with concentric rings**[a]	**Culture aroma**[b]
L. procerum	Yes	No	No	No	No	Yes	Sweet
L. truncatum	Yes	No	No	Yes	No	No	None
O. galeiformis	No	No	Yes	No	Rare	No	None
O. huntii	Yes	No	No	Yes	Yes	No	None
O. ips	No	Yes	No	No	Yes	No	None
P. fragans	No	Yes	No	No	No	Yes	Sweet

[a] Concentric rings of aerial mycelium, microconidiophores and conidia on malt extract agar

[b] Smell on malt extract agar.

Table 2.4: Distinguishing characteristics for species in the *Ophiostoma piceae* complex (Adapted from Harrington *et al*, 2001).

Species	**Colour of synnema stipe**	**Knobs on synnema stipe**	**Cupulate synnema apex**	**Synnema conidial mass**	**Colour of protoperithecia**	**Mycelium with concentric rings**[a]	**Culture aroma**[b]	**Growth (mm) at 32°C**[c]
O. piceae	Dark-brown	No	No	White	Dark-brown	Rare	None	0
O. floccosum	Red-brown	Yes	No	Yellow	Dark-brown	No	None	1-5
O. setosum	Dark-brown	No	Yes	White	Not seen	Most isolates	Sweet	0
O. querci	Dark-brown	No	No	White	Light brown	Most isolates	Nutty	5-10

[a] Concentric rings of aerial mycelium, microconidiophores and conidia on malt extract agar.

[b] Smell on malt extract agar.

[c] Extent of radial growth on malt extract agar after 7 days at 32°C.

2.4.2.2 Temperature growth assays

Temperature growth assays were used to distinguish *O. querci* from *O. piceae*. *O. piceae* was unable to grow at 32°C where as *O. querci* was capable of growth (Table 2.4) (Brasier and Stephens, 1993; Wulf and Kowalski, 1994; Harrington *et al*, 2001). Isolates were grown at 32°C on malt extract agar for seven days. The presence or absence of growth was noted.

2.4.2.3 Sexual Compatibility

For heterothallic *Ophiostoma* species, identification was often confirmed by sexual compatibility. To determine the sexual compatibility type, isolates were paired in all combinations on malt extract agar. Mycelial inocula of 2-3mm diameter were placed 2cm apart in the centre of the plate and incubated at room temperature. Perithecia formed where the two colonies met if the compatible mating type was crossed (Brasier and Kirk, 1993; Seifert *et al*, 1993). This took two to seven weeks and mating was considered successful if perithecia developed that exuded fertile ascospores.

2.5 Culture conditions

Isolates of sapstain fungi that were in constant use were maintained on agar plates, either 1.5% malt extract agar or malt yeast extract agar. Liquid cultures were prepared from actively growing cultures on agar plates. Cores were taken from the edge of the colony and transferred aseptically to 10 mL of malt yeast extract broth in a 30ml glass screw top bottle (Universal bottle). This pre-inoculum was then used to transfer fungal biomass to the inoculation medium. Inoculation medium contained 50mL of malt yeast extract broth in 250mL flasks. For larger scale inoculations to provide fungi for use in field trials, the contents of a 10mL universal starter culture were transferred to 2000mL flasks containing 1000mL of malt yeast extract broth. All flasks were incubated in a shaking incubator (Gallenkamp Orbital Incubator) at 25°C, at 125rpm, overnight for Universal flasks and three days for the 250mL and 2000mL flasks. Cultures destined for field trials were centrifuged at 25, 482g (Beckman Model J2-21M Induction Drive Centrifuge with a JA-10 rotor) for 20 minutes and the resulting supernant removed. The pellet containing mycelium and spores was resuspended in 100mM Tris-HCl pH 7 to the required spore concentration.

2.6 Storage of sapstain isolates

Sapstain fungi were stored in 20% glycerol at –70°C (Sanyo Ultralow Freezer) in the mycological culture collection at the University of Waikato, Hamilton, New Zealand. Universal flasks containing malt yeast liquid medium were inoculated with the sapstain fungal culture and incubated in a shaking incubator (Gallenkamp Orbital Incubator) at 25°C for two days. 800µL of inoculum were placed in sterile 1.5mL Eppendorf tubes with 200µL glycerol (sterilised by autoclaving for 20 minutes at 121°C), vortexed to distribute the glycerol and then stored at –70°C (Sanyo Ultralow Freezer).

2.7 Determination of fungal biomass

Two methods of fungal biomass estimation were used: haemocytometer spore counts and fungal biomass dry weight. Spore counts using a haemocytometer under a light microscope were used for *Ophiostoma* species as these fungi produced mostly spores in liquid culture. Approximately 10µL of cell suspension was transferred onto the haemocytometer and a cover slip was placed on top. All cells in 5 sub-squares of the 25 squares making up the 1mm centre square were counted. The optimum dilution for the counting was 10-20 cells per square. Each 1 mm square of the haemocytometer with the coverslip in place represents a total volume of 0.1mm^3 or 10^4cm^3. Therefore the total number of spores per mL is obtained using the following equation: number of cells x 5 x 10^4.

For fungal cultures that produced a higher proportion of hyphae the determination of biomass was measured from the dry weight. The dry weight of a liquid fungal culture was obtained by filtering on pre-weighed filters (Whatman filter paper #4) the mycelia of a known volume of culture. Filters containing mycelia were then dried to constant weight in an oven at 80°C.

2.8 Freeze drying

Cultures were grown in liquid medium (see Section 2.4), and subsequently centrifuged to remove the liquid media. The resulting cell paste was spread onto stainless steel trays and immediately placed into a -20°C freezer for one to three days. The frozen cell paste was dried in a Cuddon 1015 Fumigation freeze dryer at The Meat Industry Research Institute of New Zealand (MIRINZ), Ruakura Research Centre, Hamilton, New Zealand. The dried fungal product was put into vacuum sealed plastic bags with approximately 100gm dry weight fungus per bag and stored at –20°C until required.

2.9 Wood Specimens

All logs and wood used in this thesis for laboratory work and field trials with the exception of samples collected in Chapter 3 were *P. radiata* D. Don obtained from the Kinleith Forest, North Island, New Zealand unless otherwise stated. The trees were first generation *P. radiata* from the "850" series and originated from open pollinated seed.

2.9.1 Moisture Content analysis

The moisture content of wood samples was determined using the following equation:

$$\text{Moisture content (\%)} = \frac{\text{original weight} - \text{oven dried weight}}{\text{oven dried weight}} \times 100$$

3 Ecology and distribution of sapstain fungi in New Zealand

3.1 Introduction

A nationwide survey from 1996 to 1998 of New Zealand sapstain fungi identified the presence of *S. sapinea*, thirteen known *Ophiostoma* species and a number of undefined species of *Ophiostoma* (Farrell *et al*, 1997; Farrell *et al*, 1998). Information on this survey was presented as preliminary reports at symposia (Farrell *et al*, 1997; Farrell *et al*, 1998). These reports included lists of the sapstain species found at the time of their publication but no specific information on the ecology and distribution of each species within New Zealand. The data collected in this survey was evaluated as part of this thesis research according to ecological criteria including geographical and temporal distribution and is presented in this chapter. The data presented is the first attempt in New Zealand to link the sapstain organisms identified in New Zealand with their overall distribution within sectors of the forest industry, in different wood species and in different types of wood samples as well as to understand preferences for specific species to occupy these niches. The information generated by this survey and this thesis research is of significant benefit to the forest industry in New Zealand. In addition to the fundamental knowledge gained in this study of the major causes of sapstain, an understanding of the factors that influence sapstain populations also provides valuable information to help develop more effective control measures.

In this chapter, the hypothesis, major aims and objectives are presented first. This chapter provides methods and materials, results and discussion on the ecology of sapstain fungi in New Zealand with emphasis on the occurrence and distribution according to geographical distribution, different sectors of the forestry environment, different wood products and temporal distribution.

3.2 Hypothesis, Aims and Objectives

The hypothesis of this part of the thesis research as presented in this chapter was that the sapstain fungi of New Zealand would be preferentially found in accordance with one or more of the following criteria: geographical distribution, different sectors of the forest industry, different wood species, wood sources and season.

The major aim was to examine the distribution in New Zealand of sapstain fungi. The objectives were to compare the diversity of species found in different geographical regions of New Zealand, with an emphasis on forests, processing plants, and ports. The distribution of sapstain fungi in different wood species and wood products was investigated. Finally the relationships between sapstain fungi and temporal distributions were addressed.

3.3 Materials and Methods

3.3.1 Collection of survey data

A two-year survey, extending from Southern Hemisphere spring 1996 to winter 1998, was conducted throughout the North and South Island of New Zealand. The PhD candidate participated in this survey while a Master of Science candidate. A total of 1035 different sites were sampled. Variables in the sampling for the survey included different geographical locations, different sites within the forest environment (mills, ports, and plantations), different sample types (wood chips, general forest debris, logs, plywood, and timber), different seasons and different wood species. Samples were taken in winter (June, July, August), spring (September, October, November), summer (December, January, February), and autumn (March, April, May) for the period between spring 1996 and winter 1998. About 95% of the sampling was carried out at *P. radiata* plantation sites and the rest at native forest or non-radiata plantation sites. *P. radiata* sites included nurseries, forest plantations, urban areas, farms, mills, and ports. Some sites were re-sampled at different times of the year however the majority of sites were only sampled once during the survey.

From each site, one or more of the following methods were used to collect samples. Branches, twigs, needles, cones and wood were collected from the forest floor and bark, green needles and sapwood from the standing tree were sampled. Where possible, bark and sapwood samples from pruned or wounded areas of the tree were also taken. Samples from recently felled trees were obtained from cross section discs at least 50mm in length including both the sapwood and bark. Wood chips, timber and plywood samples were also taken. All samples were placed in clean plastic bags, sealed and used for culture isolations. The methods of culture isolation and identification of sapstain species can be found Chapter 2 (Section 2.3 and 2.4). The data obtained in the survey of

New Zealand sapstain fungi was analysed as part of the candidate's doctoral research according to the following categories:

- Geographical distribution within New Zealand (Figure 3.1).
- Forestry location (*P. radiata* plantations, processing sites, ports, non-radiata plantations, native forests and other).
- Wood source (general forest debris, wood chips, logs, plywood, seedlings, timber).
- Temporal distribution.
- Wood species.

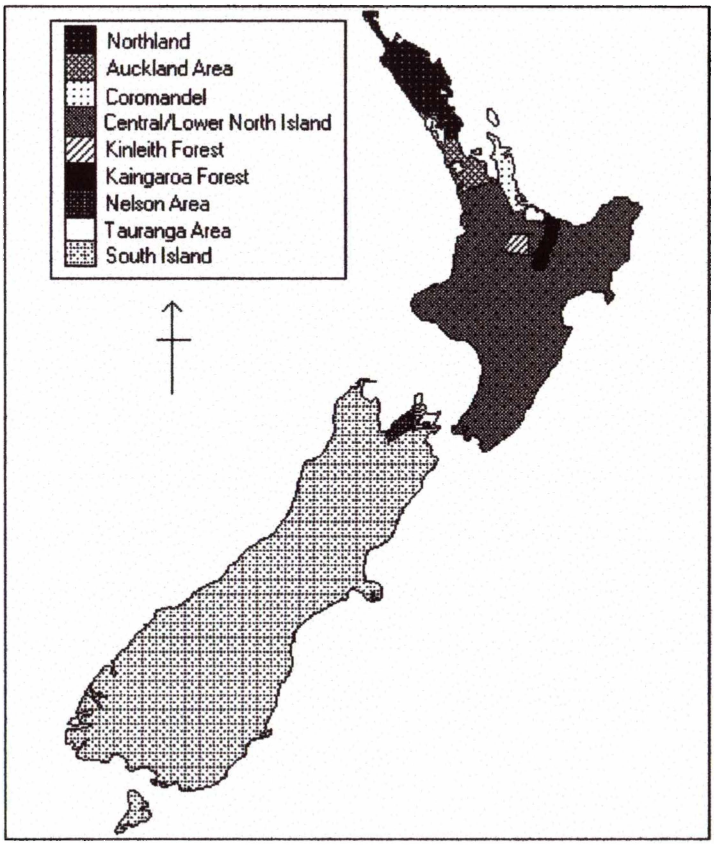


Figure 3.1: Map of New Zealand showing the geographical locations as defined for the survey.

3.3.2 Data analysis of survey of New Zealand sapstain fungi

The presence or absence of a species was recorded at each site. The occurrence of a species at a site was scored as a single record, regardless of the number of times it was isolated from that particular site or the number of colonies developed on the two media types. The proportion of individual species was recorded according to the different geographical locations, forestry location, wood source and seasons.

The following three aspects of biodiversity were evaluated; richness (S), diversity (H) and dominance. Richness was estimated as the number of different species isolated from each distinct group of sites (Lumley *et al*, 2001). Species diversity was calculated using the Shannon-Weaver index. The index was calculated using the following formula:

$$H = \Sigma_i^s P_i \ln P_i$$

Where S is the richness and P_i the portion of the total sample represented by species i. Dominance in the sapstain fungal community was judged using Carmargo's index (1/S) (Camargo, 1993) where S again represented species richness and the dominant species have the relative abundance $P_i > 1/S$ (Camargo, 1993).

3.4 Results and Discussion

3.4.1 Isolation of sapstain fungi

A total of 1035 sites were sampled during the study and a total of 1755 independent isolates of *S. sapinea* or *Ophiostoma* species were obtained. The proportions of each species and the dominant species according to Carmargo's index found in New Zealand are shown in Table 3.1. *S. sapinea* was the most commonly found fungus in this survey isolated from 54% of sites sampled within New Zealand. The most dominant *Ophiostoma* species included *O. ips*, *O. floccosum*, *O. piliferum*, *L. procerum* and *O. querci*. *O. piceae*, *O. setosum* and *O. huntii* were isolated from between 2 and 5% of sites. The least common species isolated from less than 2% of sites included *O. coronata*, *O. galeiformis*, *O. pluriannulatum*, *O. stenocerus*, *L. truncatum*, *Sporothrix* species D, *O. nigrocarpum* and *Pesotum fragans*.

The number of individual sapstain species at a site ranged from no isolations of either *S. sapinea* or members of the Ophiostomataceae family in 204 sites to ten different species of fungi found in one particular site (*P. radiata* wood chips from a pulp and paper mill in the summer period of 1997/1998). A total of 746 sites had between one and three individual species of fungi isolated at the site.

Table 3.1: *S. sapinea* and species of *Ophiostoma* isolated from New Zealand sites.

***S. sapinea* and *Ophiostoma* species**	**Frequency**	**Percentage of sites in which this species was found**
L. procerum	83[a]	8.0
L. truncatum	3	0.3
O. coronata	14	1.4
O. floccosum	218[a]	21.1
O. galeiformis	12	1.2
O. huntii	33	3.2
O. ips	299[a]	28.9
O. nigrocarpum	2	0.2
O. piceae	52	5.0
O. piliferum	146[a]	14.1
O. pluriannulatum	10	1.0
O. querci	80[a]	7.8
O. setosum	52	5.0
O. stenocerus	3	0.3
Pesotum fragans	1	0.1
S. sapinea	562[a]	54.3
Sporothrix sp. D	2	0.2

[a] Dominant species according to Carmargo's index where species is considered dominant if $P_i > 1/S$.

The dominant sapstain fungi in New Zealand were identified as *S. sapinea* and members of the Ophiostomataceae. The sapstain fungi previously reported in New Zealand and also isolated from this survey include *O. piceae* (Butcher, 1968a), *S. sapinea* (Birch, 1936), *L. truncatum* (Wingfield and Marasas, 1983), *L. procerum* (Shaw and Dick, 1980), *O. piliferum* (Hutchison and Reid, 1988a), and *O. ips*, (Hutchison and Reid, 1988a). The other eleven *Ophiostoma* species isolated in this survey had never been previously reported in New Zealand.

At the time this survey data was collected it was believed that only one member of the *Botryosphaeria* genus, *S. sapinea* was in New Zealand (Birch, 1936; Butcher, 1967; Chou, 1976a; Farrell *et al*, 1998). Recently, an unidentified *Botryosphaeria* species was found as an endophyte in *P. radiata* trees from Kinleith Forest. This fungus was distinguished from *S. sapinea* by the ability to produce pycnidia on agar media in contrast to only on cones and needles (Harrington, personal communication). In this 1996 – 1998 survey, therefore, it is uncertain whether some of the so-identified *S. sapinea* isolates might be *Botryosphaeria* species and not correctly identified. This chapter will continue to refer to *S. sapinea* isolates as *S. sapinea* but with the note that some isolates may in fact be the related *Botryosphaeria* species.

Twelve strains of *S. sapinea* isolated from the survey data and used in Kay *et al* (2002) were re-identified following the discovery of *Botryosphaeria* species in New Zealand. The isolates were also analysed by Mike Wingfield and colleagues at the University of Pretoria, Pretoria, South Africa to identify the specific morphotype of each *S. sapinea* isolate. Table 3.2 shows the re-identification and the results of morphotype analysis of the twelve isolates of *S. sapinea*. Two isolates previously described as *S. sapinea* were subsequently identified as *Botryosphaeria* species. All the *S. sapinea* isolates were of the type A morphotype. Type A isolates were described as having fluffy white to grey-green mycelia, smooth-walled conidia, lacking in microconidia, and as being pathogenic to both wounded and unwounded hosts (Swart *et al*, 1991; Swart *et al*, 1993).

Table 3.2: Re-identification of *S. sapinea* strains used in Kay *et al* (2002).

Strain number from Kay *et al* (2001)	Waikato University number	Location	Sample type	Date collected	Re-identification
HR88	D4	Dome Forest walkway, Northland	*P. radiata* cone	21/09/96	*S. sapinea* type A
HR115	D1	Mautauri Bay, Northland	*P. radiata* needles	17/09/96	*S. sapinea* type A
HR117	D12	Lake Taupo, Central North Island	*P. radiata* sapwood	28/10/96	*S. sapinea* type A
HR118	D16	Kaiaua, Coromandel	*Populus* spp. branch	9/10/96	*S. sapinea* type A
HR119	D19	Motueka Valley, Nelson	*P. radiata* needles	11/10/96	*Botryosphaeria* sp.
HR120	D21	Queenstown, South Island	*P. radiata* twig	15/10/96	*S. sapinea* type A
HR121	D30	Kaingaroa, Central North Island	*Pseudotsuga menziesii* twig	5/11/96	*S. sapinea* type A
HR122	D27	Kinleith, Central North Island	*P. radiata* sapwood	16/10/96	*S. sapinea* type A
HR123	D33	Kinleith, Central North Island	*P. radiata* sapwood	10/02/97	*S. sapinea* type A
HR152	D22	Hamilton, Central North Island	*P. radiata* cone	7/10/96	*S. sapinea* type A
HR329	D5	Mautauri Bay, Northland	*P. radiata* sapwood	27/09/96	*S. sapinea* type A
HR330	D6	Apori Forest, Northland	*P. radiata* sapwood	27/09/97	*Botryosphaeria* sp.

Both mating types of *O. piceae* and *O. querci* were identified in the survey and were widely geographically distributed. *O. piceae* and *O. querci* have both been isolated from *P. radiata* and in some cases the same pieces of *P. radiata* wood. Their co-occurrence on *P. radiata* differs from reports from the North Hemisphere where *O. piceae* is more common on conifers and *O. querci* on hardwoods (Brasier and Kirk, 1993). New Zealand *O. querci* was also isolated from *Eucalyptus* species and *Pinus taeda*.

One isolate of an unknown *Ophiostoma* species with a *Sporothrix* anamorph was isolated and subsequently named *Sporothrix* species D. This isolates would not mate with other *Ophiostoma* species isolated from New Zealand or from other fungal collections that produce only the *Sporothrix* anamorph (*O. pluriannulatum*, *O. piliferum*, *O. coronata*, *O. nigrocarpum* and *O. stenocerus*).

All the *O. coronata* isolates found in this survey failed to mate with Northern Hemisphere cultures of *O. coronata*. For convenience in this thesis the name *O. coronata* was kept but the name *Ophiostoma* species E is a more accurate representation of this species. Isolates of *Ophiostoma* species E were found on logs and timber in Canada (Uzunovic *et al*, 1999a) and were able to mate with our isolates of *O. coronata* (Harrington, personal communication).

Pesotum fragans was previously undescribed from New Zealand and isolated only once in this survey. This species was reported by Mathiesen-Käärik in Sweden from *Ips sexdentatus* gallery in a *P. sylvestris* log (Okada *et al*, 1998). ITS sequence analysis supports the inclusion of this species as *Ophiostoma* species (Harrington *et al*, 2001).

Some species reported in the literature isolated from New Zealand (Hutchison and Reid, 1988a, b) were not found in this survey and these species included: *Sphaeronaemella fimicola*, *Ceratocystiopsis falcate*, *Ceratocystis piceaperdem*, *C. novae-zelandiae*, *C. rostrocoronata* and *Hyalopesotum pini*. *Hyalopesotum pini* described by Hutchison and Reid (1988b) is a synonym of *O. galeiformis* (Harrington, personal communication). Through pairings, ITS sequence analysis and morphological features of an isolate of *Ceratocystis novae-zelandiae* (Hutchison and Reid, 1988a), Harrington (Personal communication) concluded this fungus was a synonym of *O. pluriannulatum*. Hutchison and Reid (1988a) described *Ceratocystis piceaperdem* from New Zealand and this species is morphologically very similar to *O. huntii*, but is homothallic while *O. huntii* is heterothallic. *O. piceaperdem* was not isolated in this survey of New Zealand sapstain fungi however it is thought that Hutchison and Reid (1988a) could have misidentified this species, unfortunately their cultures are unavailable to examine (Harrington, personal communication).

A total of 183 independent isolates of *Ophiostoma* species were not identified, as they were unable to be subcultured from original plates without contamination. However, they all showed recognisable features of the Ophiostomataceae: *Graphium*, *Leptographium* (*Ophiostoma* anamorphs) or perithecia (*Ophiostoma* teleomorphs) and in the majority of cases they showed the ability to grow on cycloheximide.

Various moulds belonging to the genera *Alternaria*, *Aspergillus*, *Rhizopus*, *Penicillium*, *Cladosporium*, *Geotrichum*, *Verticillium*, *Fusarium*, *Epicoccum*, *Pestalotia*, and

New Zealand (data not shown), but were not identified to species level. Dowding (1970) described these species as causing surface discolouration, invading the wood more slowly than the *Ophiostoma* species and preferring dead sapwood with high food reserves.

3.4.2 Species diversity indices

The frequency and percentages of isolation of *S. sapinea* and the total *Ophiostoma* species were calculated from the survey data. Percentages of more than 100 were obtained as more than one species of fungus was often isolated within a site. Species diversity is a common used parameter, which measures variation in species assemblages within a given community (Miller, 1995). There are a number of indices to estimate fungal species diversity including: Simpson, Shannon and McIntosh indices (Miller, 1995). The Shannon Index was chosen for this work as it was commonly used for fungal studies (Uzunovic *et al*, 1999a; Lumley *et al*, 2001). Within the Shannon Index a measure of richness was calculated, which represents the number of species within a site. Richness measurements are affected by the number of samples taken therefore all statistical analysis in this chapter relating to dominance and diversity were not entirely independent of the effect of sample size (Camargo, 1993). Dominance in the sapstain fungal community was judged using Carmargo's index (1/S) (Camargo, 1993) where S again represented species richness and the dominant species have the relative abundance $P_i > 1/S$ (Camargo, 1993).

3.4.3 Geographical distribution

Table 3.3 shows the frequency and percentage of isolations of *S. sapinea* and total *Ophiostoma* species found in the different geographical areas of New Zealand for the survey period. *Ophiostoma* species were found in high proportions in the Auckland, Coromandel, Kaingaroa, Kinleith and Northland areas. Higher amounts of *S. sapinea* than *Ophiostoma* species were isolated from sites in the South Island and the Central/Lower North Island areas. Areas of New Zealand with a high proportion of plantation forests (Auckland, Kaingaroa, Kinleith and Northland) had the highest diversity, richness and higher numbers of dominant species compared to other areas of New Zealand.

Table 3.3: The total number of isolates of *S. sapinea* and *Ophiostoma* species (percentage in parentheses) and diversity data from different geographical locations.

	Akl	C/LNI	Coro	Kga	Kin	Nel	Nthld	SI	Tga	Total
S. sapinea	221 (67.8)	36 (70.6)	71 (74.0)	30 (50.0)	103 (36.1)	16 (30.8)	37 (64.9)	31 (70.5)	17 (26.6)	562
Total *Ophiostoma* isolates	446 (136.8)	15 (29.4)	96 (100)	119 (198.3)	368 (129)	35 (67.3)	62 (108.8)	14 (31.8)	39 (60.9)	1193
Total sapstain isolates	667	51	167	149	471	51	99	45	56	1755
Total sites samples	326	51	96	60	285	52	57	44	64	1035
Richness	15	8	10	12	14	7	12	8	8	17
Diversity	2.65	1.12	1.87	3.30	2.56	1.73	2.54	1.17	1.73	2.63
Dominant species*	*S. sapinea* *O. ips* *O. floccosum* *O. piliferum* *L. procerum*	S. sapinea	*S. sapinea* *O. ips*	*O. piceae* *O. querci* O. setosum *S. sapinea* *O. ips* *O. floccosum* *O. piliferum*	*O. piceae* *O. querci* *S. sapinea* *O. ips* *O. floccosum* *O. piliferum*	*S. sapinea* *O. ips*	*S. sapinea* *O. ips* *O. floccosum* *O. piliferum* *L. procerum*	*S. sapinea*	*S. sapinea* *O. floccosum*	

Akl: Auckland area, C/LNI: Central/ Lower North Island, Coro: Coromandel, Kga: Kaingaroa Forest, Kin: Kinleith Forest, Nel: Nelson, Nthld: Northland, SI: South Island, Tga: Tauranga. * Dominant according to Carmargo's index.

This project resulted in the first large-scale detailed survey of sapstain fungi across New Zealand. This study showed conclusively the following:

1. There were no obvious differences in sapstain fungal species from different geographical locations throughout the North and South Islands of New Zealand.
2. Richness and diversity were greatest in areas of New Zealand with plantation forestry.

3.4.4 Distribution in different forestry environments

The frequency and percentages of positive isolations of *Ophiostoma* species and *S. sapinea* for different forestry environments, processing sites and other areas are shown in Table 3.4. Non-radiata plantations (any exotic plantation not including *P. radiata*) had approximately equal amounts of *Ophiostoma* species to *S. sapinea*. More *Ophiostoma* species were found compared to *S. sapinea* in *P. radiata* plantations and processing sites (including ports). Native forests and others areas (including nurseries, urban and farm areas) had relatively high proportions of *S. sapinea*.

The richness and diversity of species were greatest in sites that were related to the forest industry (*P. radiata* and non-*P. radiata* plantations and processing site). A number of dominant fungi were isolated from *P. radiata* plantations and processing sites including *S. sapinea* and *Ophiostoma* species. Members of the *O. piceae* complex (*O. piceae*, *O. querci*, *O. floccosum* and *O. setosum*) as well as *S. sapinea* and *O. ips* were dominant at processing sites. In *P. radiata* plantation sites *O. piliferum*, *L. procerum*, *O. floccosum*, *O. ips* and *S. sapinea* were dominant.

Table 3.4: The total number of isolates of *S. sapinea* and *Ophiostoma* species (percentage in parentheses) and diversity data from location type.

	Processing sites	**Native Forest**	**Non-radiata plantation**	**Other**	**Port**	***P. radiata* plantation**	**Total**
S. sapinea	59 (40.1)	14 (58.3)	15 (62.5)	22 (55.0)	12 (21.4)	440 (59.1)	562
Total *Ophiostoma* isolates	207 (140.8)	2 (8.3)	16 (66.7)	15 (37.5)	33 (58.9)	920 (123.7)	1193
Total sapstain isolates	266	16	31	37	45	1360	1755
Total sites samples	147	24	24	40	56	744	1035
Richness	12	3	10	9	8	16	17
Diversity	2.76	0.58	2.04	1.49	1.52	2.62	2.63
Dominant species*	*O. piceae* *O. querci* *O. setosum* *S. sapinea* *O. ips* *O. floccosum*	*S. sapinea*	*S. sapinea* *O. ips*	*S. sapinea*	*S. sapinea* *O. floccosum*	*S. sapinea* *O. floccosum* *O. ips* *O. piliferum* *L. procerum*	

* Dominant according to Carmargo's index.

Native forest areas had the lowest diversity (0.58) and there were only three species isolated with *S. sapinea* as a dominant fungus. Again as discussed in Section 3.5.2, the lack of vectors of sapstain fungi could be the reason for the low diversity in native forests. This finding has great relevance to biosecurity concerns for New Zealand native forest and bush areas.

3.4.5 Distribution in various wood products

Table 3.8 shows the number and percentages of positive isolations of *S. sapinea* and *Ophiostoma* species from general forest sampling, seedling and other wood products. *S. sapinea* was more prominent in the general forest samplings (needles, cones and other forest debris) than the *Ophiostoma* species. Logs, timber, wood chips and plywood all had more *Ophiostoma* species than *S. sapinea.* Diversity was greatest in logs, timber and wood chips. Richness was greatest in logs, general forest samples and wood chips. Wood chips had the largest number of dominant species with nine species including *S. sapinea* and *Ophiostoma* species. *S. sapinea* was dominant in all sample types except seedlings, which had no dominant sapstain fungi.

Table 3.5: The total number of isolates of *S. sapinea* and *Ophiostoma* species (percentage in parentheses) and diversity data from different sample types.

	Wood chips	General forest samples	Logs	Plywood	Seedlings	Timber	Total
S. sapinea	6 (35.3)	126 (72.4)	411 (52.5)	2 (50.0)	3 (30.0)	14 (29.8)	562
Total *Ophiostoma* isolates	49 (288.2)	88 (50.6)	1013 (129.4)	3 (75.0)	1 (10.0)	39 (83.0)	1193
Total sapstain isolates	55	214	1424	5	4	53	1755
Total sites samples	17	174	783	4	10	47	1035
Richness	11	12	16	4	2	7	17
Diversity	3.76	1.75	2.69	1.39	0.59	1.96	2.63
Dominant species*	*O. piceae* *O. querci* *O. setosum* *O. ips* *S. sapinea* *O. floccosum* *O. huntii* *L. procerum* *L. truncatum* *O. piliferum*	*S. sapinea* *O. floccosum*	*O. querci* *S. sapinea* *O. ips* *O. piliferum* *O. floccosum* *L. procerum*	*S. sapinea*	No dominant species	*S. sapinea* *O. ips* *O. floccosum*	

* Dominant according to Carmargo's index.

The spores of *S. sapinea* are associated with cones and needles (Palmer *et al*, 1988) that are found more commonly on the forest floor. *S. sapinea* sporulates prolifically on cones, after they have opened and discharged seeds (Peterson, 1977; Chou, 1984). The inoculum produced on these retained cones plays an important role in the survival of the fungus. The conidia produced on these cones may infect current year shoots and also the second year seed cones. The inoculum density of *S. sapinea* is higher therefore in the forest environment. The total incidence of *Ophiostoma* species was found to be higher in *P. radiata* plantations, at processing sites and ports, as well as in wood chips, timber and logs than *S. sapinea*. The sticky spores on the synemma and perithecial stalks of *Ophiostoma* species, are disseminated by wind and insect vectors, and are commonly found on logs and timber (Dowding, 1970). The insect vectors, particularly the bark beetles are found both in logs and stumps (Reay and Walsh, 2001). Insect and wind increases the spread of *Ophiostoma* species from logs processing sites and ports, therefore more inoculum potential for *Ophiostoma* species should occur at these sites.

3.4.6 Temporal Distribution

Table 3.6 represents the frequency and percentage of isolates of *S. sapinea* and total *Ophiostoma* species found in the different seasons of this survey. *S. sapinea* was slightly more prevalent in spring and summer than winter and autumn. Proportions of

Ophiostoma are higher in the autumn and winter than in spring and summer. Richness and diversity were greatest in Winter 1997 with values of 14 and 3.32 respectively.

Table 3.6: The total number of isolates of *S. sapinea* and *Ophiostoma* species (percentage in parentheses) and diversity data from different seasons.

	Spring 96	**Summer 96/97**	**Autumn 97**	**Winter 97**	**Spring 97**	**Summer 97/98**	**Autumn 98**	**Winter 98**	**Total**
S. sapinea	37(69.8)	65 (75.6)	70 (45.8)	79 (53.4)	81 (63.8)	139 (57.0)	79 (40.3)	12 (42.9)	562
Total *Ophiostoma* isolates	18 (34.0)	34 (39.5)	161 (105.2)	257 (173.6)	123 (96.9)	212 (86.9)	355 (181.1)	33 (117.9)	1193
Total sapstain isolates	55	99	231	336	204	351	434	45	1755
Total sites samples	53	86	153	148	127	244	196	28	1035
Richness	9	11	11	14	12	11	11	4	17
Diversity	1.32	1.37	2.41	3.32	2.35	2.17	2.48	1.28	2.63
Dominant species*	*S. sapinea*	*S. sapinea* *O. floccosum*	*O. setosum* *S. sapinea* *O. ips* *O. floccosum*	*O. piceae* *O. querci* *O. setosum* *S. sapinea* *O. ips* *O. floccosum* *O. piliferum* *O. huntii* *L. procerum* *O. coronata*	*S. sapinea* *O. ips* *O. floccosum* *O. piliferum* *L. procerum*	*S. sapinea* *O. ips* *O. floccosum* *O. piliferum* *L. procerum*	*O. querci* *S. sapinea* *O. ips* *O. floccosum* *O. piliferum*	*S. sapinea* *O. ips*	

* Dominant according to Carmargo's index.

S. sapinea was found more in spring and summer than total *Ophiostoma* species, while *Ophiostoma* species were more abundant in the autumn and winter months (March to August).

Reay *et al* (2002) described the significant relationship between sub-lethal attack of seedlings by the introduced bark beetle *Hylastes ater* and the invasion by many sapstain fungi. The flight activity of *H. ater* is predominantly in the autumn months in New Zealand (Reay and Walsh, 2001). The relationship between the presence of the greater numbers of *Ophiostoma* species in autumn and the flight activity of *H. ater* maybe related and needs further investigation. The increased diversity and richness of sapstain fungi in areas of New Zealand with major plantations could also be attributed to the spread of *Ophiostoma* species by the bark beetle *H. ater*. *H. ater* was suggested as the mechanism by which a number of species of sapstain fungi were introduced to New Zealand (Wingfield and Gibbs, 1991; Jacobs *et al*, 1998). *H. ater* is predominately a vector in second rotation forests since the insects nest in stumps produced by harvesting (Reay *et al*, 2002), therefore areas with first rotation plantation are unaffected or minimally affected by this bark beetle.

Brookhouser and Peterson (1971) described the dissemination of *S. sapinea* spores only during periods of rainfall and at an optimum temperature of 24°C for germination. In this survey there were more isolations of *S. sapinea* in spring and summer but this fungus was also isolated in the winter and autumn. Swart and Wingfield (1991) found that spring was the most infectious time for *S. sapinea* into pruning wounds, relating to higher temperatures prevailing after periods of maximum rainfall.

3.4.7 Wood species

Every species of sapstain fungi isolated in New Zealand was found on *P. radiata* but only some of the species of sapstain fungi were found on native and other exotic wood types (Table 3.7).

Table 3.7 Sapstain species isolated from wood species tested.

Sapstain species	Wood species
L. procerum	*Pinus radiata, Pinus taeda*
L. truncatum	*Pinus radiata*
O. coronata	*Pinus radiata*
O. floccosum	*Pinus radiata*
O. galeiformis	*Pinus radiata*
O. huntii	*Pinus radiata, Pinus taeda*
O. ips	*Acer negundo* (Box elder), *Pinus radiata, Pinus taeda*
O. nigrocarpum	*Pinus radiata, Pinus taeda*
O. piceae	*Pinus radiata*
O. piliferum	*Liriodendron tulipifera, Pinus radiata, Pinus taeda*
O. pluriannulatum	*Eucalyptus* sp., *Pinus radiata, Pinus taeda, Nothofagus solandri var. solandri* (Black beech)
O. querci	*Eucalyptus sp., Pinus radiata, Pseudotsuga menziesii* (Douglas fir)
O. setosum	*Pinus radiata, Pinus taeda, Pseudotsuga menziesii* (Douglas fir)
O. stenocerus	*Pinus radiata*
Pesotum fragans	*Pinus radiata*
S. sapinea/ Botryosphaeria sp.	*Acer negundo* (Box elder), *Cordyline australis* (Cabbage tree), *Pseudotsuga menziesii* (Douglas fir), *Eucalyptus* sp., *Liriodendron tulipifera, Cypressus lusitanica, Pinus maritima, Populus* species, *P. radiata, Chamaecyparis lawsonia, Magnolia grandiflora*
Sporothrix sp. D	*Pinus radiata*

Investigations within New Zealand, into the causes of sapstain have mainly been associated with exotic softwoods, particularly *P. radiata* (Butcher, 1968a). *O. pluriannulatum* was isolated from this survey on Black beech (*Nothofagus solandri* var. *solandri*). Butcher (1968b) isolated *O. piceae* from red beech and silver beech. Hutchison and Reid (1988a) found *O. coronata* on *Eucalyptus* species, and *O. piceae* from *Dacrydium cupressinum* (Rimu), *Eucalyptus* species and Douglas fir.

The presence of *S. sapinea* on a number of hardwoods was unexpected as this fungus is predominately found on coniferous species (Peterson, 1977). As previously mentioned *S. sapinea* may have been misidentified in this survey. It is therefore uncertain whether the isolation of *S. sapinea* from the hardwood species was in fact *Botryosphaeria* species. *Botryosphaeria* species commonly occur on hardwood species (Jacobs and Rehner, 1998).

Each individual type of sapstain species was isolated from *P. radiata*. Whether these fungi were brought to New Zealand on this wood species or on bark beetles now associated with *P. radiata* is unsure. Either way, all of the sapstain fungi have adapted to *P. radiata* wood as their niche and major nutrient source in New Zealand.

The following descriptions describe the ecological data pertaining to each individual *Ophiostoma* species and *S. sapinea*. These include findings on the geographical distribution, temporal occurrence, distribution at different locations, within the different sample types and on specific wood species. This is the first time these species have been described in New Zealand in this detail regarding ecological niche. Others in the location category include urban areas and nurseries.

***Ophiostoma* species within the *O. piceae* complex**

***O. floccosum* (Figure 3.2)**

Occurrence: Auckland, Central North Island, Coromandel, Kaingaroa, Kinleith, Nelson, Northland, South Island, Tauranga
Season: Autumn, Spring, Summer, Winter
Location: Processing sites, Others, Port, *P. radiata* plantations
Sample type: Wood chips, General debris samplings, Logs, Timber
Wood species: *P. radiata*

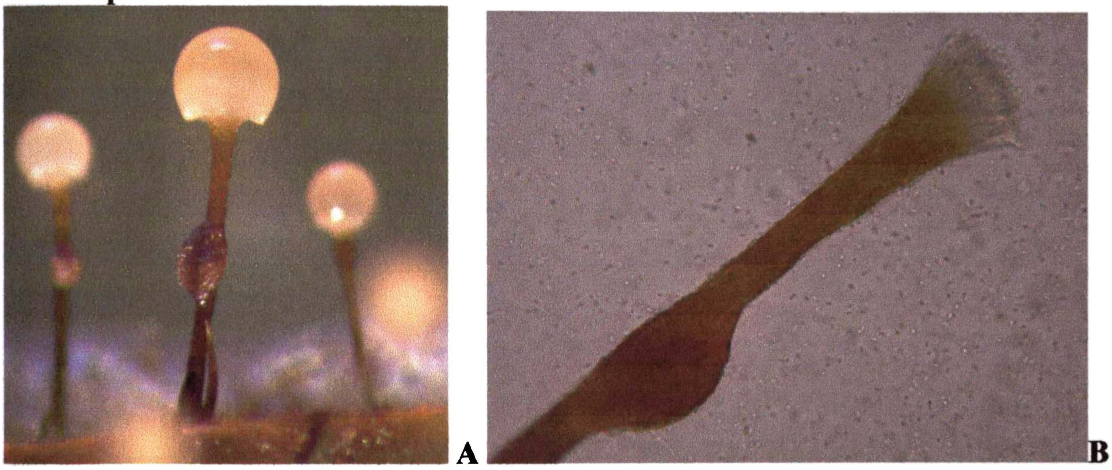


Figure 3.2: Synnemata of *O. floccosum* A= 50X magnification; B=400X magnification.

O. piceae

Occurrence: Auckland, Central North Island, Coromandel, Kaingaroa, Kinleith, Nelson, Northland, South Island, Tauranga
Season: Autumn, Spring, Summer, Winter
Location: Processing sites, Native forests, Non-radiata plantations, Others, Port, *P. radiata* plantations
Sample type: Wood chips, General debris samplings, Logs, Timber
Wood species: *P. radiata*

O. querci (Figure 3.3)

Occurrence: Auckland, Central North Island, Coromandel, Kaingaroa, Kinleith, Nelson, Northland, South Island, Tauranga
Season: Autumn, Spring, Summer, Winter
Location: Processing sites, Non-radiata plantations, Others, Port, *P. radiata* plantations
Sample type: Wood chips, General debris samplings, Logs, Plywood, Timber
Wood species: Douglas fir (*Pseudotsuga menziesii*), *P. radiata*, *Eucalyptus* sp.

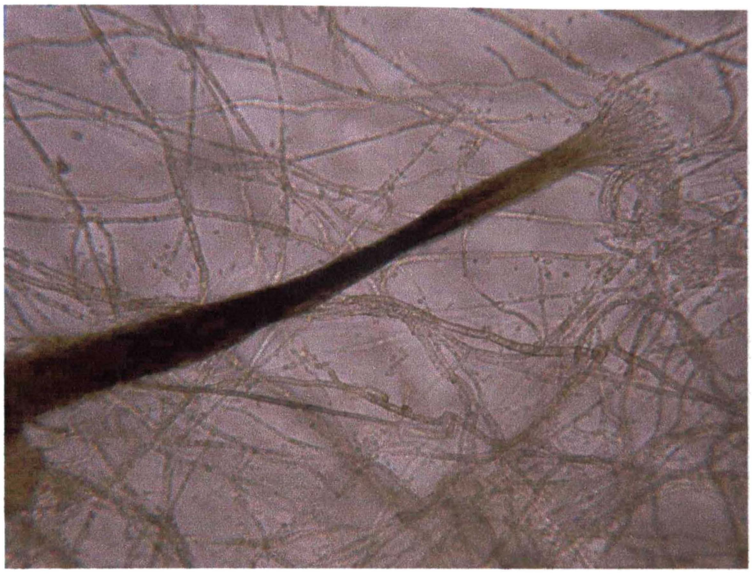


Figure 3.3: Synnemata of *O. querci* 400X magnification

O. setosum **(Figure 3.4)**

Occurrence: Auckland, Central North Island, Coromandel, Kaingaroa, Kinleith, Nelson, Northland, South Island, Tauranga
Season: Autumn, Spring, Summer, Winter
Location: Processing sites, Non-radiata plantations, Others, Port, *P. radiata* plantations
Sample type: Wood chips, General debris samplings, Logs, Plywood, timber
Wood species: Douglas fir (*Pseudotsuga menziesii*), *P. radiata*, *P. taeda*

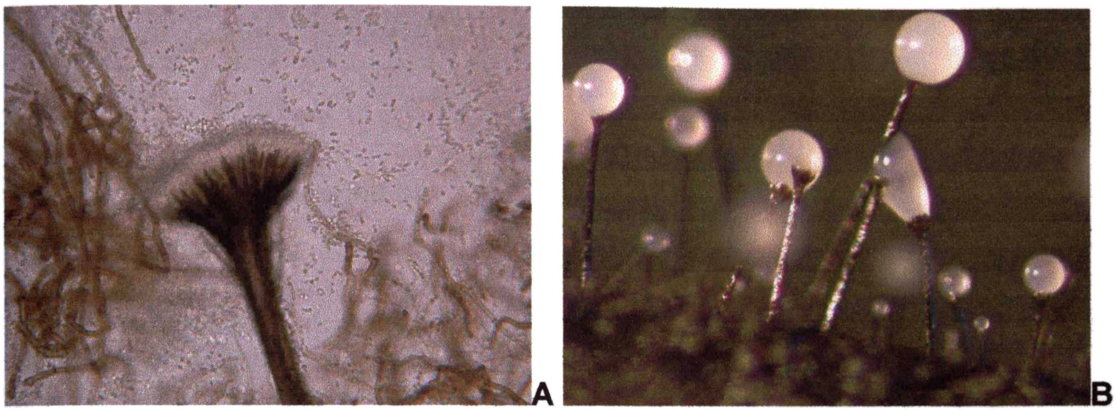


Figure 3.4: Cupulated synnemata of *O. setosum* A= 400X magnification; B =20X magnification

Species with *Pesotum* anamorph only

Pesotum fragans

Occurrence: Kinleith
Season: Spring
Location: Processing sites
Sample type: Wood chips
Wood species: *P. radiata*

O. piliferum

Occurrence: Auckland, Central North Island, Coromandel, Kaingaroa, Kinleith, Nelson, Northland, Tauranga.
Season: Autumn, Spring, Summer, Winter
Location: Processing sites, Non-radiata plantations, Others, Port, *P. radiata* plantations
Sample type: Wood chips, General debris samplings, Logs, Timber
Wood species: *Liriodendron tulipifera*, *P. radiata*, *P. taeda*

O. pluriannulatum (Figure 3.5)

Occurrence: Auckland, Kaingaroa, Kinleith, South Island
Season: Autumn, Summer, Winter
Location: Processing sites, Native forest, Non-radiata plantations, *P. radiata* plantations
Sample type: General debris samplings, Logs
Wood species: *Eucalyptus* sp., *P. radiata*, *P. taeda*, Black beech (*Nothofagus solandri* var. *solandri*)

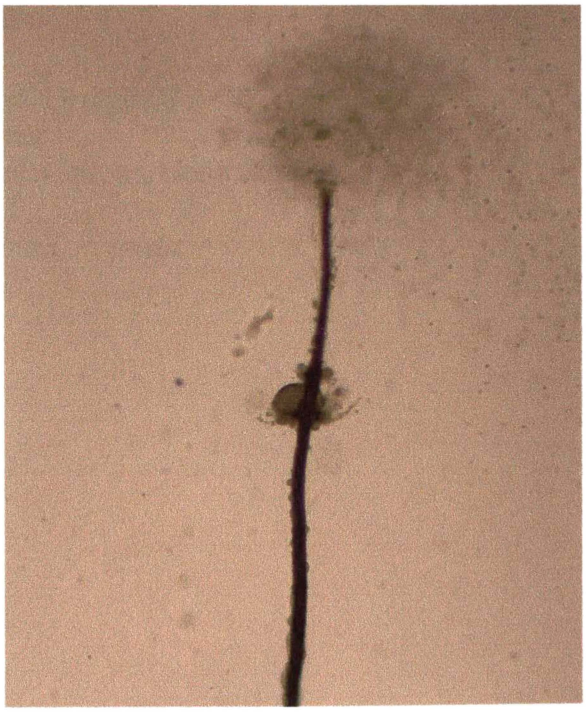


Figure 3.5: Apex of perithecia of *O. pluriannulatum* (400X) showing annulations and conidia release at tip.

O. coronata (*Ophiostoma* species E)

Occurrence: Auckland, Kinleith, Northland
Season: Autumn, Spring, Winter
Location: Others, *P. radiata* plantations
Sample type: General debris samplings, Seedlings, Logs
Wood species: *P. radiata*

O. stenocerus

Occurrence: Central North Island, Kinleith, Northland
Season: Spring, Summer
Location: Others, *P. radiata* plantations
Sample type: General debris samplings, Logs
Wood species: *P. radiata*,

Sporothrix species D

Occurrence: Auckland
Season: Winter
Location: *P. radiata* plantations
Sample type: Logs
Wood species: *P. radiata*

O. nigrocarpum

Occurrence: Auckland, Kaingaroa
Season: Autumn, Winter
Location: *P. radiata* plantations, Non-radiata plantation
Sample type: Logs
Wood species: *P. radiata, P. taeda*

L. procerum (Figure 3.6)

Occurrence: Auckland, Central North Island, Coromandel, Kaingaroa, Kinleith, Northland, South Island, Tauranga
Season: Autumn, Spring, Summer, Winter
Location: Processing site, Non-radiata plantations, Others, *P. radiata* plantations
Sample type: Wood chips, general debris samplings, Logs, Timber
Wood species: *P. radiata*, *P. taeda*

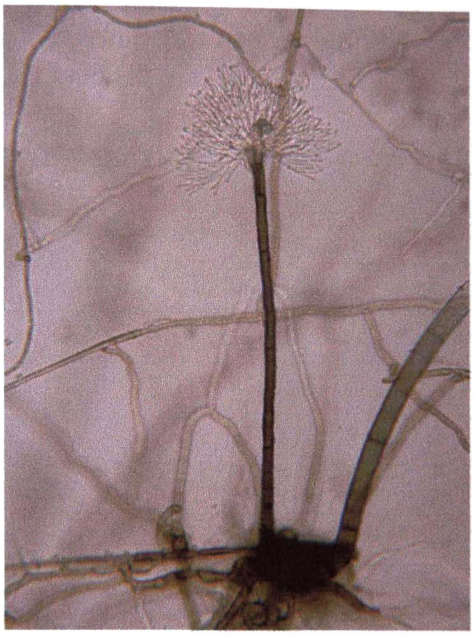


Figure 3.6: The *Leptographium* structure of *L. procerum* under 400X magnification.

L. truncatum

Occurrence: Auckland, Kaingaroa
Season: Spring, Winter
Location: Processing sites, *P. radiata* plantations
Sample type: Wood chips, Logs
Wood species: *P. radiata*

O. huntii (Figure 3.7)

Occurrence: Auckland, Coromandel, Kaingaroa, Kinleith, Northland, South Island, Tauranga
Season: Autumn, Spring, Summer, Winter
Location: Processing sites, Non-radiata plantations, Port, *P. radiata* plantations
Sample type: Wood chips, General debris samplings, Logs, Plywood
Wood species: *P. radiata*, *P. taeda*

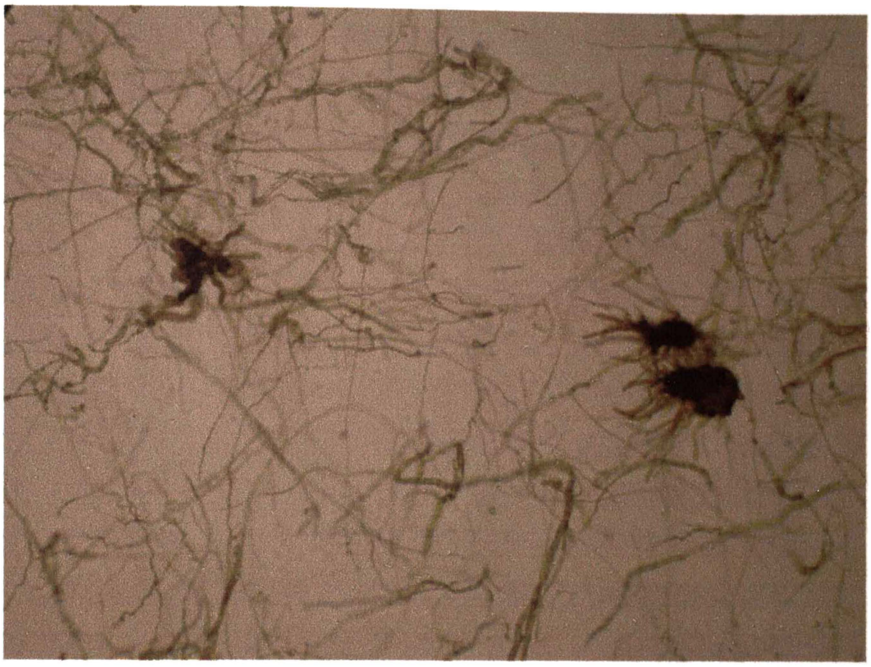


Figure 3.7: ***O. huntii*** **showing characteristic serpentine hyphae under 100X magnification.**

Other groups of *Ophiostoma* species

O. ips

Occurrence: Auckland, Central North Island, Coromandel, Kaingaroa, Kinleith, Nelson, Northland, Tauranga
Season: Autumn, Spring, Summer, Winter
Location: Processing sites, Non-radiata plantations, Port, *P. radiata* plantations
Sample type: Wood chips, General debris samplings, Logs, Timber
Wood species: Box elder (*Acer negundo*), *P. radiata*, *P. taeda*

O. galeiformis (Figure 3.8)

Occurrence: Auckland, Coromandel, Kinleith, Northland
Season: Autumn, Spring, Summer, Winter
Location: *P. radiata* plantations
Sample type: Logs.
Wood species: *P. radiata*

Figure 3.8: *Pesotum* anamorph structure of *O. galeiformis* under 400X magnification.

***S. sapinea/Botryosphaeria* species (Figure 3.9)**

Occurrence: Auckland, Central North Island, Coromandel, Kaingaroa, Kinleith, Nelson, Northland, South Island, Tauranga

Season: Autumn, Spring, Summer, Winter

Location: Processing sites, Native, Non-radiata plantations, Others, Port, *P. radiata* plantations

Sample type: Wood chips, General debris samplings, Logs, Plywood, Seedlings, Timber

Wood species: Box elder (*Acer negundo)*, cabbage tree (*Cordyline australis*), Douglas fir (*Pseudotsuga menziesii*), *Eucalyptus* species, *Liriodendron tulipifera*, *Cypressus lusitanica*, *Pinus maritima*, *Populus* species, *P. radiata*, *Chamaecyparis lawsonia*, *Magnolia grandiflora*.

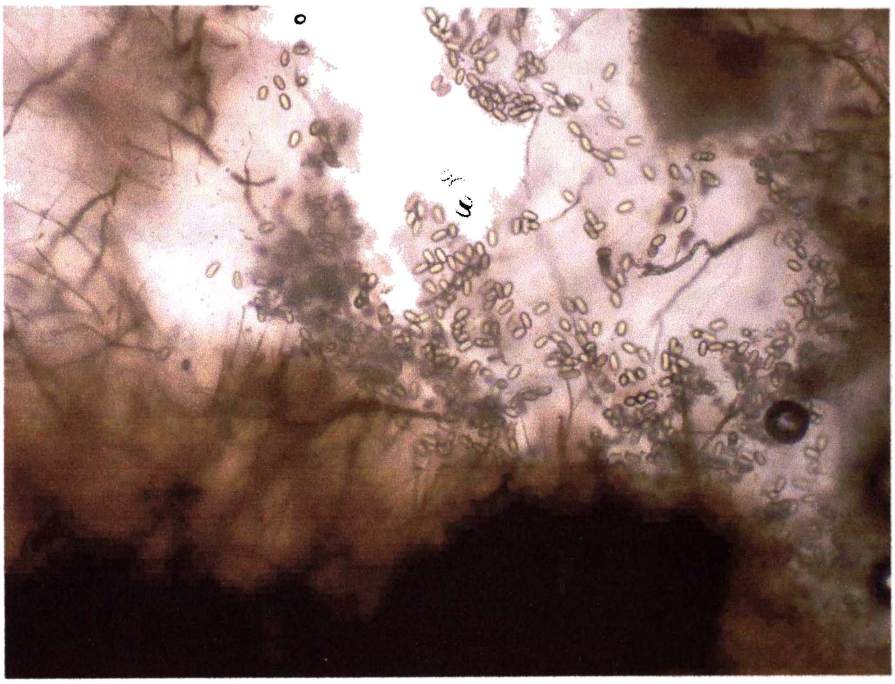


Figure 3.9: ***Botryosphaeria* species conidia release from pycnidia under 400X magnification.**

3.4.8 Summary and conclusions

There are very few detailed surveys measuring the distribution of sapstain fungi in different geographical regions, within different seasons and different wood sources and species worldwide (Uzunovic *et al*, 1999a). There are two main reasons for this. Firstly, knowledge about the taxonomy of particularly the *Ophiostoma* species requires complex morphological studies to adequately identify the fungi to species level. With morphological analysis and increased knowledge of the *Ophiostoma* species within New Zealand it was easier to identify these fungi (Harrington *et al*, 2001). Secondly, a survey of the sapstain fungi within a country requires extensive and organised sampling (Uzunovic *et al*, 1999a). The survey described in this thesis research was undertaken with the assistance of the two major Forestry companies in New Zealand, Fletcher Challenge Forests and Carter Holt Harvey Forests. With the companies help this survey was able to extensively sample major forest plantations within the North and South Island of New Zealand over a two-year period.

The results of this study showed that there were no obvious differences in sapstain fungal species associated with different geographical locations throughout the North and South Island of New Zealand. The climatic variation within New Zealand therefore did not affect the species of sapstain fungi. The climatic variation was also studied by looking at the affect of season on individual sapstain species. There was individual sapstain species predominated in a particular seasons, however as mentioned previously, the proportions of *S. sapinea* and the total number of *Ophiostoma* species were affected by season.

This study found that *S. sapinea* was the most widely distributed and the major cause of sapstain in the survey endorsing the results of Birch (1936) and Butcher (1967). In this study, *S. sapinea* was isolated throughout New Zealand, in all seasons and at all stages from felling to port and processing operations. Chou (1984) also found that *S. sapinea* was widely distributed throughout New Zealand. Kay *et al* (2002) examined twenty isolates of *S. sapinea* from this survey, for colony and morphological characteristics, and pathogenicity to *P. radiata* seedlings. Variation was evident in the S. sapinea isolates and they could not be placed in the previously described morphotypes (Kay *et al*, 2002). The results of the study by Kay *et al*, (2002) indicated a shift in the New Zealand *S. sapinea* population, either reflecting a re-introduction, spread of additional isolates or a shift in the structure of the resident population. This thesis research showed that two of the isolates previously described by Kay *et al* (2002) as *S. sapinea* were *Botryosphaeria* species and the other *S. sapinea* isolates were all of the type A morphotype.

It is important to note that the species dominance does not necessary indicate the staining ability of the fungi. Not all *Ophiostoma* species growing on wood are economically important sapstain fungi nor in fact do all the species stain wood. Hutchison and Reid (1988a) tested the staining ability of the *Ophiostoma* species they isolated from New Zealand. *O. coronata* produced no stain on any of the wood tested (Hutchison and Reid, 1988a). *O. piceae* was not considered to be an important sapstain organism (Lagerberg *et al*, 1927; Bakshi, 1951; Hutchison and Reid, 1988a). Other reports, however suggested that *O. piceae* produces a penetrating light grey stain (Seifert, 1993). Due to the taxonomic discrepancies with this species, it is unsure whether these reports were studying *O. piceae* or another member of the *O. piceae* complex. In contrast, species like *O. piliferum* (Seifert, 1993), *S. sapinea* (Butcher, 1967), and *O. ips* (Seifert, 1993) produce extensive grey to black stain on wood. The staining ability of many of the *Ophiostoma* species isolated in this survey was tested in this thesis research. Sapstain species were grown on *P. radiata* specimens in the laboratory and were assessed for stain development. These results are given in Chapter 5 (Section 5.5.3).

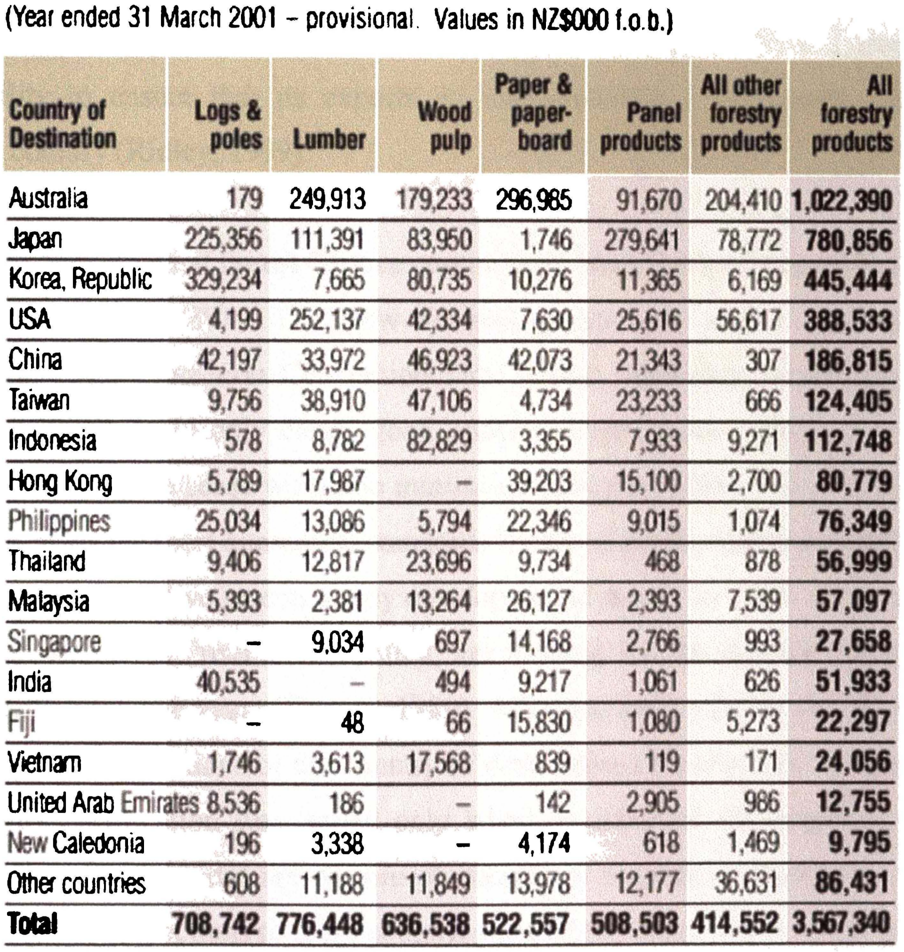
In conclusion, seventeen individual species of *Ophiostoma* and *S. sapinea* were isolated from the survey of New Zealand sapstain fungi including one unnamed species. No sapstain fungi predominated in any geographical area of New Zealand. The diversity of sapstain fungi was greatest in areas with high proportions of plantation forests. More *Ophiostoma* species compared to *S. sapinea* were cultured from *P. radiata* plantations and processing sites, however, native forest areas and other areas including nurseries, urban areas and farm areas had relatively high proportions of *S. sapinea*. *S. sapinea* was more prominent in the general forest samplings (needles, cones and other forest debris) than the *Ophiostoma* species. From logs, timber, wood chips and plywood, more *Ophiostoma* species than *S. sapinea* were cultured. *S. sapinea* was found more in spring and summer, while *Ophiostoma* species were more abundant in the autumn and winter months. A number of wood species were sampled in this survey including native and exotic wood species. All sapstain species were isolated from *P. radiata* and a number of sapstain fungi were isolated from other wood species.

Knowledge of taxonomy and ecology of the sapstain fungi in New Zealand from this survey assisted in the discovery of more effective control methods for stain reduction. Biological control methods with albino technology using colourless strains of the most common sapstain fungi in New Zealand are presented in Chapter 6.

4 Sapstain fungi on *Pinus radiata* logs – from New Zealand Forest to Export Destinations in Japan

4.1 Introduction

New Zealand is one of the worlds' largest exporters of softwood logs. Exports of forest products provide 4% of New Zealand's gross domestic product (GDP) with the major markets for logs including Japan, Korea, USA and Philippines (New Zealand Forest Owners, 2001) (Figure 4.1). The shipment of *P. radiata* from New Zealand to these export destinations requires lengthy transport times and crossing the equator where moist warm conditions are often encountered making prevention of sapstain crucial.



(Year ended 31 March 2001 – provisional. Values in NZ$000 f.o.b.)

Country of Destination	Logs & poles	Lumber	Wood pulp	Paper & paper-board	Panel products	All other forestry products	All forestry products
Australia	179	249,913	179,233	296,985	91,670	204,410	**1,022,390**
Japan	225,356	111,391	83,950	1,746	279,641	78,772	**780,856**
Korea, Republic	329,234	7,665	80,735	10,276	11,365	6,169	**445,444**
USA	4,199	252,137	42,334	7,630	25,616	56,617	**388,533**
China	42,197	33,972	46,923	42,073	21,343	307	**186,815**
Taiwan	9,756	38,910	47,106	4,734	23,233	666	**124,405**
Indonesia	578	8,782	82,829	3,355	7,933	9,271	**112,748**
Hong Kong	5,789	17,987	–	39,203	15,100	2,700	**80,779**
Philippines	25,034	13,086	5,794	22,346	9,015	1,074	**76,349**
Thailand	9,406	12,817	23,696	9,734	468	878	**56,999**
Malaysia	5,393	2,381	13,264	26,127	2,393	7,539	**57,097**
Singapore	–	9,034	697	14,168	2,766	993	**27,658**
India	40,535	–	494	9,217	1,061	626	**51,933**
Fiji	–	48	66	15,830	1,080	5,273	**22,297**
Vietnam	1,746	3,613	17,568	839	119	171	**24,056**
United Arab Emirates	8,536	186	–	142	2,905	986	**12,755**
New Caledonia	196	3,338	–	4,174	618	1,469	**9,795**
Other countries	608	11,188	11,849	13,978	12,177	36,631	**86,431**
Total	**708,742**	**776,448**	**636,538**	**522,557**	**508,503**	**414,552**	**3,567,340**

Figure 4.1: New Zealand exports of wood and wood products by destination (source New Zealand Forest Owners, 2001).

Japan, as the Asia Pacific region's largest importer of forest products, is expected to maintain its dominant consumption and importing position in most wood product markets for the next 10 years (Ogle and Miller, 2000). Wood supply in Japan depends on imports from various parts of the world including North America, New Zealand, Chile, Europe and China. Most export wood requires treatment at Japanese sawmills with anti-sapstain formulations to supply 'clean' unstained wood to Japanese markets (Tsunoda and Kumagai, 1999).

As global movement of wood and wood products increases so does the threat of the introduction of non-indigenous fungal species. Wingfield *et al* (2001) described that little is known about the intercontinental spread of pathogens that infect solid wood, especially the sapstain fungal pathogen *S. sapinea* which is now widespread through exotic pine plantations. Wingfield *et al* (2001) stated that *Ophiostoma* species have also spread from native pine-growing areas to exotic plantations. New Zealand has a responsibility to ensure that its exports do not constitute a biosecurity risk to the importing country (Ridley, 1999).

In 1948, the New Zealand Forest Service commenced intensive import/export quarantine operations (Cooper, 1989). The New Zealand government was a signatory to the International Plant Protection Convention 1951 which supported the principle that if exporting countries sold only clean, good quality forest produce the inter-continental spread of insects and disease would be minimised (Cooper, 1989). Prior to 1989, New Zealand set its own export quarantine standards for forest products and prohibited export of produce containing live insects at any developmental stage. In 1986, the Government decided that, under its "user pays" policy, net funding for the forestry service would reduce to zero after five years (Cooper, 1989). After this date, exports merely needed to meet the specific regulations of the country of destination (Butcher and Dysdale, 1991). Phytosanitary certification was issued only when required by the importing country. Japan, Korea and China did not require phytosanitary inspection certificates for sawn timber or logs, so inspections to these countries ceased (Cooper, 1989). At entry ports in Japan and Korea fumigation or some other form of sterilisation is automatically carried out. Other importing countries demand phytosanitary certification based on the International Plant Protection Convention format (Cooper, 1989).

In 1997, a group of American environmental organizations successfully sued the Animal and Plant Inspection Service (APHIS) of the United Stated Department of Agriculture (USDA) over APHIS's regulations allowing the importation of non-tropical raw logs and wood chips into the United States. The court issued a nationwide injunction prohibiting the issue of further import permits until the USDA adequately disclosed the biological risks that such importations posed to American forests (Ridley, 1999). In January 1999, the United States District Court in San Francisco cancelled the prohibition on permit issues. However, New Zealand has now developed a set of protocols to minimise the chance that *P. radiata* export logs to the Unites States carry any undesirable organisms (Ridley, 1999). The protocols include carrying out regular systematic surveys, maintaining a centralised database of forest biota, and by undertaking quality export timber inspections (Ridley, 1999).

In New Zealand logs can be stored at the wharf for up to two months (Butcher and Dysdale, 1991). By inspecting logs prior to shipment, it is possible to ensure that they are free from insect infestation and fungal infection, but this does not guarantee the condition of the material at the point of delivery (Butcher and Drysdale, 1991). Passage through warm, moist tropical conditions is conducive for fungal development, and therefore deterioration is believed to occur during transportation of logs offshore to Northern Hemisphere countries. Export logs are protected from deterioration in New Zealand by debarking and anti-sapstain treatment (Butcher and Drysdale, 1991). The removal of bark reduces the risk of insect infestation, as many of the bark beetles (Scolytidae) require the presence of bark for attack (Reay, 2000). Bark removal, however, does not completely remove the risk of pest infestation. Control of sapstain in New Zealand and worldwide, is accomplished by fungicide treatment with a variety of chemical active ingredients including: copper-8-hydroxy-quinolinolate, chlorothanonil, carbendazim, didecyl dimethylammonium chloride (DDAC) and methylene bis thiocyanate (MBT) (FRI, 1997; Morrell and Xiao, 1999). A more extensive review of chemical control of sapstain fungi is provided in Chapter 6 (Section 6.3.1.2).

In places with temperate climates like New Zealand, the length of unprotected exposure of logs in the forest after harvesting is crucial with regard to sapstain infection. Unless the logs are processed shortly after harvesting, there is usually a high risk of fungal infection (Butcher and Dysdale, 1991). Delays in processing result in the localised loss of moisture, especially from log ends and areas where bark is damaged or removed during extraction.

In New Zealand, a mathematical model (Sapstain Danger Index, SDI) was developed and validated which is used to indicate forest sites where harvested logs are more susceptible to sapstain infection (Zeff, 1999; Cooper *et al*, 2000). This index also calculates how soon after harvesting a log should be treated in order to minimise sapstain development, by using parameters such as temperature, rainfall and relative humidity. Cooper *et al* (2000) showed that in five sites monitored in the Central North Island of New Zealand, there was a correlation between the Sapstain Danger Index and the number of days until sapstain infection occurred.

Lee and Gibbs (1996) investigated the influence of harvesting machinery and chainsaws on the development of sapstain. Machine harvested logs had significantly more stain than chainsaw harvested logs and this was directly correlated with the amount of bark removed or loosened. The use of spiked rollers resulted in more stain than when rubber rollers were used. Uzunovic *et al* (1999b) also observed that less bark was damaged when rubber feed rollers were used in contrast to the use of metal spiked rollers. Intact bark is thought to give effective and prolonged protection against fungal damage.

This chapter reports on the distribution of sapstain fungi from harvesting to an export destination (Japan) on untreated and anti-sapstain treated logs. It is uncertain for *P. radiata*, which fungal species contribute to the major sapstain problem at the each stage of production from harvesting to an export destination. Two field trials were established, one in New Zealand summer and the other in New Zealand winter. No other trials that the author is aware of were published that follow logs from harvesting to export destinations especially the movement from the Southern Hemisphere to the Northern Hemisphere.

4.2 Aims and Objectives

The aim of this study was to verify in a controlled and measured environment the ecological patterns of sapstain colonisation at specific points in the processing of logs from harvesting to an export destination. This study also compared the effect of shipment in two different seasons (New Zealand summer and winter) and monitored environmental conditions during storage and shipment of the logs. A comparison of the time of harvest to anti-sapstain treatment and the effects of anti-sapstain treatment on sapstain colonisation were also addressed.

4.3 Material and Methods

Two trials were established in different climatic conditions, New Zealand summer and New Zealand winter, of generally the same design where mature harvested logs were treated with anti-sapstain chemical, shipped to the same export destination, Japan and evaluated for their physical appearance and fungal species present.

4.3.1 Export Field Trial I - Summer 2001

This field trial was established on the 7th February 2001 at a storage yard in Kinleith Forest, Central North Island, New Zealand. Freshly harvested logs from approximately 24-year-old trees, with an average diameter of 27cm, with lengths of approximately 6.1 metres, were sourced from Kinleith Forest. There were a total of 91 logs in the summer trial. Treatments were separated into two groups, the first compared non-anti-sapstain treated logs and anti-sapstain treated, and the second group compared the time of harvest to anti-sapstain treatment.

Group 1. Treatment 1 (No anti-sapstain treatment)
Treatment 2 (Anti-sapstain treated 4 days post-harvest)
Group 2. Treatment 3 (Anti-sapstain treated 4 days post-harvest)
Treatment 4 (Anti-sapstain treated 1 day post-harvest)

Logs were debarked just prior to anti-sapstain treatment. For Treatment 1, logs were not debarked as this represented the normal export logs to Japan at the time of establishment of this trial and had no anti-sapstain treatment. Chemical treatments occurred either at one day or four days post-harvest and consisted of logs being sprayed with a benchmark anti-sapstain product Cutrol 375, (distributed by Fernz Timber Protection, PO Box 88-048, Clendon Town, Auckland, New Zealand) at a concentration of 9.5 % w/v. Cutrol 375 contains Copper-8-quinolinolate as the active ingredient. The concentration level of this product was determined by the industry and was justified on the basis of cost and efficacy. A pink dye was also added to ensure good coverage validation of the anti-sapstain chemical. The logs were stored in the Kinleith trial area until transportation to the Port of Tauranga (Figure 4.2) and shipment to Japan. The Export Field Trial I logs went to the Port of Akita, Northern Japan (Figure 4.2).

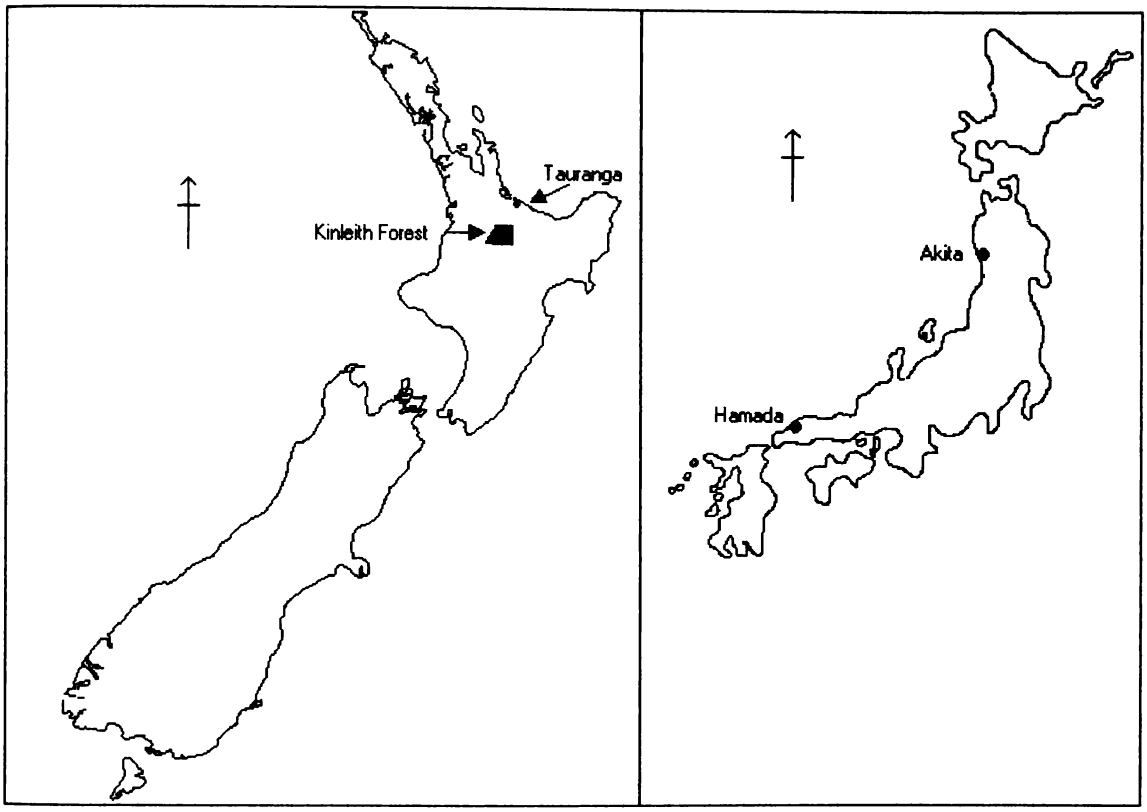


Figure 4.2: Maps of New Zealand and Japan, showing harvesting sites (Kinleith Forest area) the Port of export (Tauranga) in New Zealand and Ports of import in Japan (Akita, Export Field Trial I and Hamada, Export Field Trial II).

The logs were assessed for appearance and sampled for fungal isolations at three time points: immediately before anti-sapstain treatment, at five weeks just prior to transportation to the port for loading aboard the ship, and at ten weeks after arrival at the Port of Akita, Japan. All logs were sampled at each time points as follows; a 0.5 metre section was sliced from both ends of each log and discarded. The 50mm sample disc was then sliced from both ends of all logs. Each disc was assessed for visual sapstain coverage and fungal infection. Two independent assessors estimated the amount of visual sapstain on the face of each disc using the scale in Table 4.1. Only shades of blue, grey or black stain were used in the estimation of stain.

Table 4.1: Visual stain categories used in the Export Field Trials to estimate the amount of sapstain coverage on disc faces.

0 = no stain (0%)
1 = Minimally stained (1-10%)
2 = Mildly stained (11-20%)
3 = Moderately stained (21-50%)
4 = Heavily stained (51-80%)
5 = Severely stained (81–100%)

Sapstain fungal infection was determined from five randomly sampled pieces taken from each disc. For each time point the time between sampling and processing in the laboratory varied. For the first two time points (before anti-sapstain treatment and prior to shipment) the samples were all processed within two days of sampling. There was a delay in processing the samples from Japan due to transportation of the samples back to New Zealand. The Japan samples were stored when possible at 5°C and were processed within one week of sampling. The pieces were surface sterilised at room temperature by soaking in 5% hypochlorite solution for approximately two minutes and rinsed twice in sterile water. The samples were then slivered with a sterile scalpel and slivers placed onto two selective media: Media 4 and Media 6 as described more fully in Chapter 2 Section 2.3).

The selective media plates with wood slivers were incubated for up to 30 days at 25°C in a darkened chamber. All sapstain fungi growing on the selective media plates were aseptically transferred onto fresh agar plates, containing 1.5% malt extract agar as they developed. Cultures of *S. sapinea* and *Ophiostoma* species were identified as previously described in Chapter 2 (Section 2.4).

4.3.2 Export Field Trial II - Winter 2001

Export Field Trial II was established on the 28th of August 2001 (New Zealand winter) at the same location as the first trial with logs sourced from Kinleith Forest. *P. radiata* trees were felled, cut and treated as previously described with the exception of the control logs which were debarked due to concerns in the first trial of potential beetle infestation of the bark. The treatments were the same as the first logs. Again the logs were stored in the Kinleith trial area until transportation to the Port of Tauranga (Figure 4.2) and shipment to Japan in the MV RUBIN FOREST voyage number V60A. The Export Trial II logs were shipped to the Port of Hamada, Southern Japan (Figure 4.2).

Logs were assessed for sapstain discolouration and sampled for fungal infection immediately before anti-sapstain treatment, prior to transportation to Japan (8 weeks post-harvest) and at the Port of Hamada, Japan (15 weeks post-harvest). Logs were analysed for visual stain coverage and sampled for fungal colonisation using the methods described in Export Field Trial I.

4.3.3 New Zealand Parallel Trial

An identical trial was established at the same time as Export Field Trial II with identical treatments and is referred to as the New Zealand Parallel Trial. This trial served to compare logs exported to Japan with logs left in New Zealand with regards to physical appearance of sapstain and fungi present on the logs. These logs were left at the storage site in Kinleith and assessed once, fourteen weeks post-harvest.

4.3.4 Data analysis

Statistical analysis of the visual stain was performed using the program Minitab Version 12. Individual logs from each treatment were considered replicates. Differences with respect to severity of stain between treatments and time of sampling were investigated using analysis of variance (ANOVA). Comparisons were conducted using Tukey's pairwise comparisons to determine the nature of the differences detected by ANOVA.

For each time point, the presence or absence of an individual species on the selective media was recorded. The occurrence of species in a sample was scored as a single record regardless of the number of colonies developing on the media. The percentage of isolations for each species at each time point and for each treatment was calculated as the number of positive isolations of a species/total isolation attempts x 100 (P_i). Values of species richness were used to evaluate diversity. Species richness was estimated as the number of different species per site. Dominance of fungal species on a treatment or at a specific time period were analysed using Carmargo's index (1/S) where S represented species richness (the number of competing species in the community) and a species was termed dominant if $P_i > 1/S$ (Camargo, 1993).

4.3.5 Climatic Information

Weather information, both mean daily rainfall and temperature, for the trial periods at Kinleith were provided by a weather station within the area. Two climatic data recorders (Hobo ® H8 Pro RH/Temperature loggers) accompanied the logs on both ships for Trial I and Trial II, the NIN and MV RUBIN FOREST respectively, with one data logger placed above deck and one placed below deck. Measurements of temperature and relative humidity were recorded.

4.4 Results and Discussion

4.4.1 Climatic Information

Climatic information for the time periods when the logs were stored at Kinleith and aboard the ships during transportation was measured for Export Trial I, II and the New Zealand Parallel Trial. Figure 4.3 and Figure 4.4 show the mean daily temperature and rainfall for the time period that the logs were held at Kinleith from the 7th February to the 8th March 2001 for Export Field Trial I and 28th August to the 28th November for Export Field Trial II and the New Zealand Parallel Trial. Trial I had an average temperature of 18°C with a range from 13-22°C, for the period of harvesting and holding of the logs pre-shipment at Kinleith. The average rainfall for this period was 7mm but rainfall during this period occurred on only seven days with the other 24 days having no rainfall.

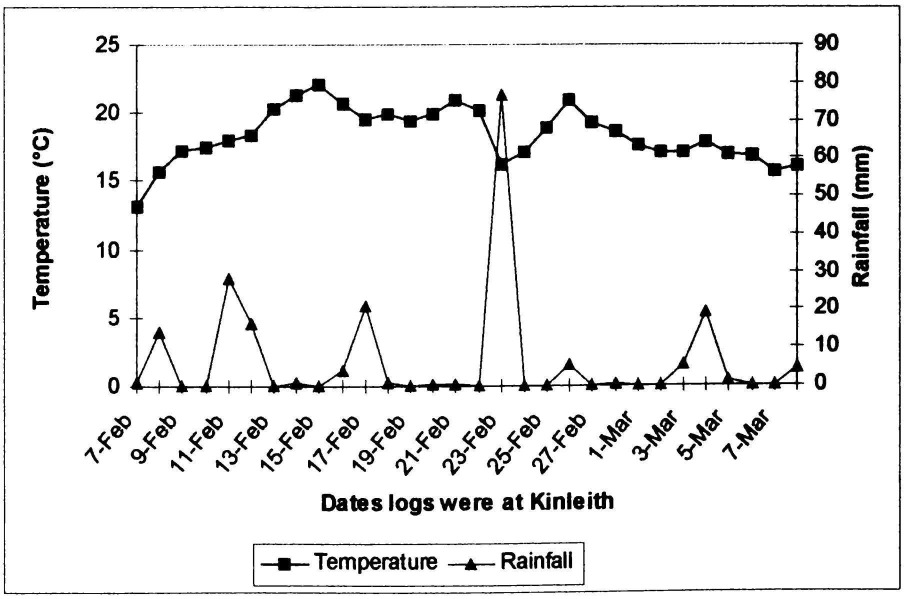


Figure 4.3: Mean daily temperatures and rainfall for Export Field Trial I, the period from harvesting to pre-shipment at Kinleith (7th February 2001- 8th March 2001).

Export Field Trial II (between the 28th August and the 25th October) had significantly lower temperatures and rainfall than the summer Export Field Trial I. Export Field Trial II had an average temperature of 11°C and average daily rainfall of 3mm for the period from harvesting to shipment of the export logs. The temperatures and rainfall were measured for the period following shipment of Export Field Trial II to monitor the weather conditions for the back up trial. An average temperature of 13°C and average daily rainfall of 8mm was recorded for this period from the 26th October to the 28th November. A total of 19 days during this period had significant rainfall. This period thus had more rainfall and the temperatures were slightly warmer than the previous period.

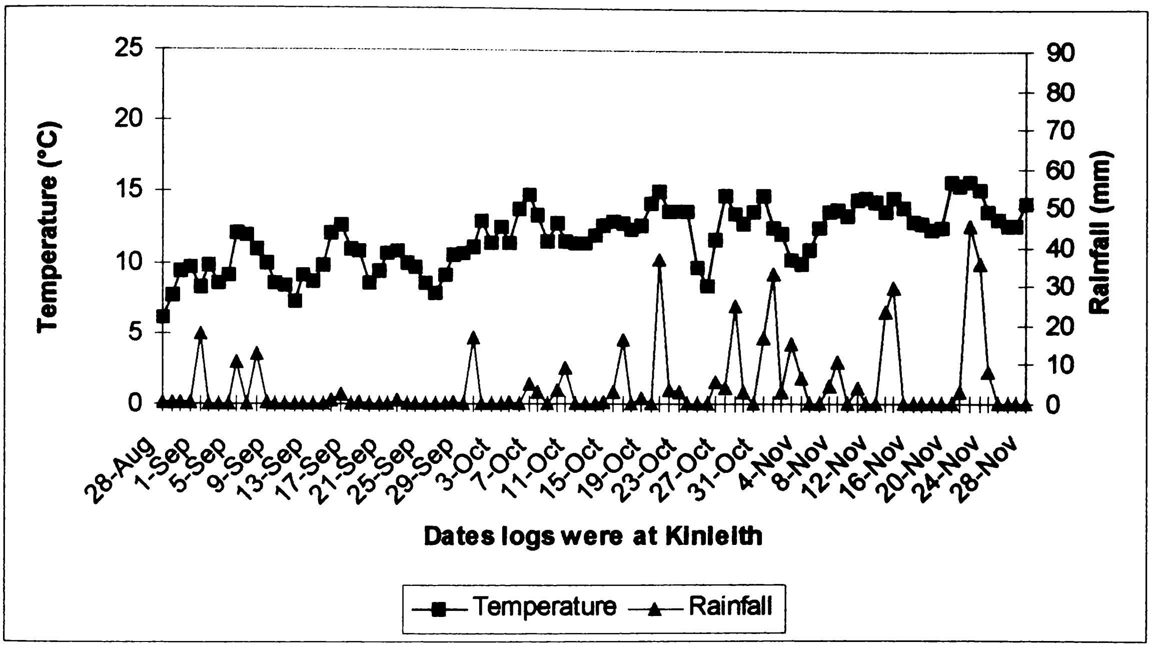


Figure 4.4: Mean daily temperatures and rainfall for Export Field Trial II and the New Zealand Parallel Trial, for the period from harvesting to pre-shipment and post-shipment at Kinleith (28th August 2001- 28th November 2001).

Two climatic data recorders, one above deck and one below deck measured mean daily temperature and relative humidity for the duration of the shipment to Japan in both trials (Figure 4.5 and Figure 4.6). For Trial I, the logs were aboard the ship NIN from the 21st of March to 10th April 2001. The temperatures and relative humidity readings recorded were less varied below deck. The temperatures ranged from 11 to 28 °C above deck and 17 to 25°C below deck. The relative humidity ranged from 61-95% above deck and 75-96% below deck.

For Export Field Trial II, the logs were aboard the MV RUBIN FOREST from the 27th October to 28th November. The temperatures were slightly lower for this trial, compared to Export Field Trial I. The temperatures ranged from 10 to 29°C above deck and 13 to 22°C below deck. Relative humidity was approximately the same for Export Field Trials I and II. Relative humidity for the shipment period of this trial ranged between 56 to 92% above deck and 79-99% below deck.

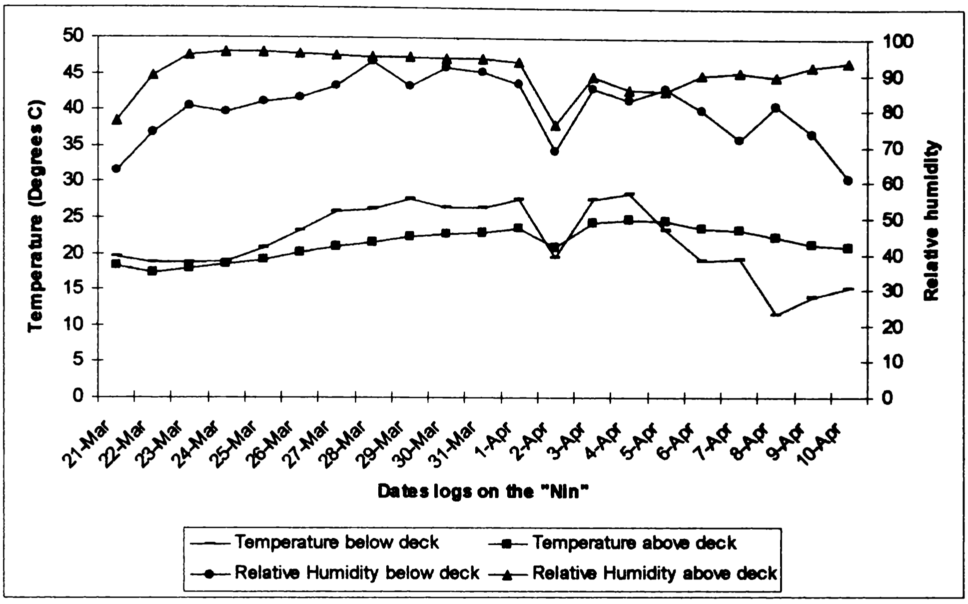


Figure 4.5: Mean daily temperature and relative humidity above and below deck of the ship NIN for the period of shipment during Export Field Trial I (21st March 2001-10th April 2001).

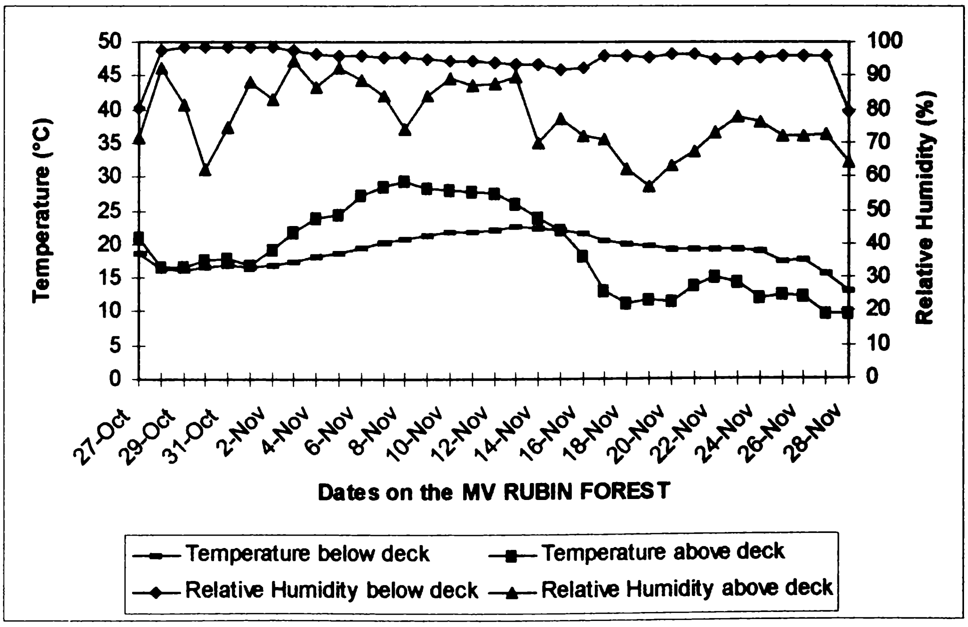


Figure 4.6: Mean daily temperature and relative humidity above and below deck of the MV RUBIN FOREST for the period of shipment during Export Field Trial II (27th October 2001- 28th November 2001).

4.4.2 Visual Stain data

The patterns of sapstain discolouration evident on the *P. radiata* logs were typically wedge shaped. The stained wedges radiated inward from the cambium and were in some cases associated with puncture marks on the outer surface of the logs (Figure 4.7).

Figure 4.7: Patterns of stain evident in Export Field Trial I at the sampling in Akita, Japan. A: log treated with anti-sapstain within one day of harvesting (stain associated with a puncture mark on outer surface. B: Log treated with anti-sapstain chemical four days post-harvest (stain in wedge shape).

Visual stain data for both trials according to sampling period is provided in Table 4.2. No logs were stained on any treatment prior to anti-sapstain treatment for either trial. Export Trial I (summer) had more stained logs than Export Trial II (winter) prior to shipment to Japan. The visual stain on logs pre-shipment ranged from minimal (0-10%) to heavily stained (51-80%) in Export Field Trial I compared to Export Trial II where the majority of stained logs were only minimal (0-10%) (Figure 4.8). Logs arriving in Japan that were harvested in New Zealand summer had more stained logs and more severity of stain than logs harvested in winter.

At the same time as Export Trial II, a second set of logs with identical treatments to Export Trial II was also established and subsequently called New Zealand Parallel Trial. This set of logs was set up to compare logs at the export destination to logs left in New Zealand and the logs were only sampled once, a week before the logs exported to Japan were sampled. The logs left in New Zealand had an average severity of stain of 1.0 compared to the logs exported to Japan, which had an average severity of stain of 0.2. There were also a number of mildly and moderately stained logs in the New Zealand

Parallel Trial, whereas the logs exported to Japan were only minimally stained. This shows that trials in New Zealand do not mimic what is happening to logs that are exported.

Table 4.2: The number of logs grouped according to the values of stain severity for the different sample times.

	Before AST		Before Shipment		Japan		New Zealand Parallel	
	Trial I	Trial II	Trial I	Trial II	Trial I	Trial II	Trial I	Trial II
No stain	91	92	3	58	9	46	No data	20
Minimally stained (0-10)	0	0	56	33	28	9	No data	54
Mildly stained (11-20)	0	0	21	1	27	0	No data	14
Moderately stained (21-50)	0	0	9	0	22	0	No data	4
Heavily stained (51-80)	0	0	2	0	5	0	No data	0
Severely stained (81+)	0	0	0	0	0	0	No data	0
Average severity of stain	0	0	1.5	0.4	1.8	0.2	No data	1.0
Total number of logs	91	92	91	92	91	55	No data	92

Figure 4.8: Logs from Export Field Trial I and II, both logs treated with anti-sapstain chemical four days post-harvest and sampled in Japan. A: Export Field Trial I (Moderately stained) B: Export Field Trial II (No stain).

Visual stain data for both trials according to the four different treatments is shown in Table 4.3. There was a statistical difference in the average severity of stain between, Treatment 1 (non anti-sapstain treated logs) and Treatment 2 (logs treated with anti-sapstain four days post-harvest) for Trial I (F= 8.7, P=0.003) and Trial II (F=33.5, P<0.001) (Figure 4.9). In both Trial I and Trial II, the non anti-sapstain treated log had more severe staining than the anti-sapstain treated logs. Treatment 4 (the logs treated one day post-harvest) was less severely stained compared to Treatment 3 (the logs treated after four days) for Trial I (F=36.7, P<0.001) (Figure 4.9). However, for Trial II (New Zealand winter) there were significant differences between Treatment 4 and Treatment 3 (F=0.3, P=0.616).

Table 4.3: The number of logs grouped according to the values of stain severity for the different treatments.

	Treatment 1 No anti-sapstain		Treatment 2 Anti-sapstain 4 days		Treatment 3 Anti-sapstain 4 days		Treatment 4 Anti-sapstain 1 day	
	Trial I	Trial II	Trial I	Trial II	Trial I	Trial II	Trial I	Trial II
No stain	23	27	26	56	22	65	32	74
Minimally stained (0-10)	14	32	32	33	7	26	31	18
Mildly stained (11-20)	18	21	11	2	17	1	2	0
Moderately stained (21-50)	9	12	3	1	18	0	1	0
Heavily stained (51-80)	5	0	0	0	2	0	0	0
Severely stained (81+)	0	0	0	0	0	0	0	0
Average severity of stain	1.4	1.2	0.9	0.4	1.6	0.3	0.5	0.2
Total number of logs	69	92	72	92	66	92	66	92

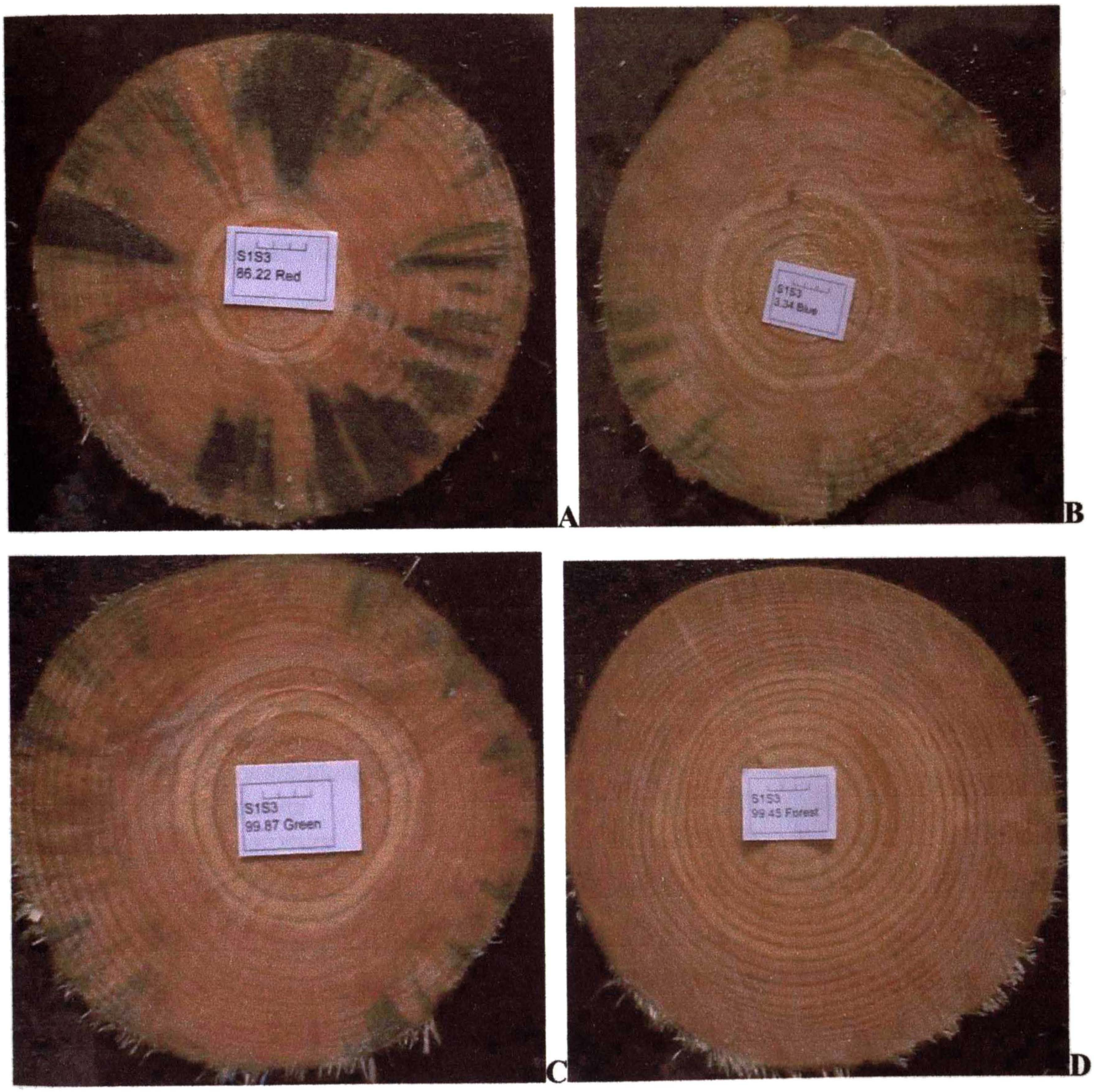


Figure 4.9: Logs from Export Field Trial I at Akita, Japan each with a different treatment. A: No anti-sapstain chemical. B: Anti-sapstain chemical applied 4 days after harvesting. C: Anti-sapstain chemical applied 4 days after harvesting. D: Anti-sapstain chemical applied one day after harvesting.

4.4.3 Fungal data analysis

From the 2730 samples taken, a total of 878 individual sapstain fungi were isolated in the Export Trial I established at the end of New Zealand summer 2001. Both *S. sapinea* and eight *Ophiostoma* species were isolated and identified (Table 4.4). The most commonly isolated fungi were *O. floccosum*, *O. querci* and *O. setosum*. A number of *Ophiostoma* species grew on the selective media but were unable to be obtained in pure culture for identification. These species were identified as *Ophiostoma* species due to morphological features (synnemata and perithecia) evident on the media as well as their ability to grow in the presence of cycloheximide and are given in Table 4.4 as *Ophiostoma* sp. In comparison, Export Field Trial II and the New Zealand Parallel Trial both established at the same time in winter 2001 (the only difference between the two trials is Trial II logs were shipped to Japan and New Zealand parallel logs remained in New Zealand) had a total of 451 individual sapstain fungi isolated from 3680 samples taken. The most common sapstain fungi were *S. sapinea*, *O. querci*, *O. floccosum*, *O. setosum* and *O. piceae* (Table 4.4). In both trials sapstain fungi were isolated from both stained and unstained pieces of wood.

Table 4.4: Overall percentage (and frequency) of individual sapstain species isolated from Export Field Trials I and II.

Sapstain species	Trial I*	Trial II*
L. procerum	1.7 (45)	0.1 (3)
Ophiostoma sp.	4.3 (118)	0.3 (12)
O. floccosum	8.8 (241)	1.4 (50)
O. huntii	1.9 (52)	0.1 (4)
O. ips	0.4 (10)	not identified
O. piceae	1.5 (40)	1.3 (48)
O. piliferum	0.01 (1)	0.1 (3)
O. pluriannulatum	not identified	0.03 (1)
O. querci	7.0 (192)	2.7 (98)
O. setosum	4.5 (122)	1.4 (51)
S. sapinea	2.1 (57)	4.93 (181)
Total independent sapstain isolates	32.2 (878)	12.3 (451)
Total pieces of wood sampled	2730	3680

*numbers in brackets are the frequency of sapstain species collected

Other wood inhabiting fungi grew on the selective media, including *Alternaria* species, *Cladosporium* species, *Epicoccum* species, *Fusarium* species, *Mucor* species, *Penicillium* species, *Pestalotia* species and *Trichoderma* species. These fungi were not characterised to species level, as the scope of the project was to identify the penetrating sapstain species. Dowding (1970) described these species as causing only surface discolouration, invading the wood more slowly than the *Ophiostoma* species and preferring dead sapwood with high food reserves. *Trichoderma* species were also isolated in both trials but were also evident on the outer surfaces of the samples from the time point in Japan for Export Field Trial I prior to processing in New Zealand. *Trichoderma* species are described as fast growing primary colonisers of wood capable of utilising the sugar present and are able to survive for long periods of time under field conditions (Bruce *et al*, 2000).

Table 4.5 shows the frequency of each species isolated at each time point for Export Field Trial I. *O. querci*, *O. floccosum* and *O. setosum* were all isolated in high numbers before anti-sapstain treatment. These fungi were colonising the wood within four days of harvesting. A variety of *Ophiostoma* species and *S. sapinea* were isolated in high numbers prior to shipment (five weeks post-harvest). At the Port of Akita, Japan, the sapstain fungi *O. floccosum*, *O. querci*, *O. setosum* and *O. huntii* were isolated in high numbers. *O. huntii* was only isolated once before shipment. Isolations of *O. floccosum* and *L. procerum* increased from harvesting to the export destination.

Table 4.5: Frequency of sapstain fungi isolated in the sapwood of *P. radiata* from harvest to export destination for Export Field Trial I.

	Location		
	Before AST	**Before shipment**	**Akita, Japan**
L. procerum	7	9	29
Ophiostoma sp.	42	33	43
O. floccosum	40	87	114
O. huntii	0	1	51
O. ips	0	3	7
O. piceae	16	8	16
O. piliferum	1	0	0
O. querci	77	68	47
O. setosum	42	39	41
S. sapinea	20	35	2
Total	245	253	323

The increased isolations of *Ophiostoma* species compared to *S. sapinea* indicated that the *Ophiostoma* species might out compete *S. sapinea* over time. Chapter 6 (Section 6.5.9) shows results of albino *O. floccosum* producing a metabolite that stops the growth of *S. sapinea* on synthetic media.

At the Port of Akita, Japan, the sapstain fungus *O. huntii* was isolated in high numbers, compared to the other sampling periods. From this data *O. huntii* appeared to be a secondary coloniser, predominately being isolated when the logs were held after harvesting for more than nine weeks.

Table 4.6 shows the percentage of wood samples with sapstain fungi isolated at each sampling period and for each treatment for the trials. The effect of season was evident in the amount of sapstain fungi isolated when comparing Export Field Trial I (summer) and Export Field Trial II (winter). There were higher amounts of sapstain fungi isolated from the logs before anti-sapstain treatment for Export Field Trial I than Export Field Trial II. This trend was also seen at the sampling point prior to shipment and at the ports in Japan. The New Zealand Parallel Trial logs had less fungi isolated compared with the logs exported to Japan. These logs, however, were more stained than the logs exported to Japan. It is unsure why less fungi was isolated, one reason maybe that the logs exported to Japan were colonised with fungi that were not melanised. The effect of environmental conditions and the production of melanisation are discussed in Chapter 5 (Section 5.5.2.3).

An increase in isolations of sapstain fungi occurred over time for each treatment. Treatment 1, no anti-sapstain treatment logs had more isolations of sapstain fungi than the anti-sapstain treated logs at the two time points post the application of anti-sapstain treatment. The timing of anti-sapstain treatment after harvesting was significant. When the logs were treated with anti-sapstain within one day of harvesting there were less isolations of sapstain fungi at subsequent time points, with the exception of Export Field Trial II, time point in Japan. These logs had no visual sapstain but sapstain fungi were isolated from 23% of the samples.

Table 4.6: Percentage of wood samples with sapstain fungi isolated at each sampling period and treatment for Export Field Trial I and II.

Treatment	Percentage of wood samples with sapstain fungi isolated							
	Sampling Period							
	Before AST		Before shipment		Japan		New Zealand Parallel	
	Trial I	Trial II	Trial I	Trial II	Trial I	Trial II	Trial I	Trial II
Treatment 1	33	0.9	50.4	10.4	85.6	No data*	No data	27.4
Treatment 2	28.3	3.5	17.9	6.1	25.4	17.7*	No data	23
Treatment 3	15.9	5.7	20.9	3.9	12.7	19.6	No data	17
Treatment 4	30	2.6	12.7	0	29	23.9	No data	8.7

*Some logs were not shipped to Japan, these logs were sampled in New Zealand at the same time as the back up logs, with the following values Control = 20.4% and AST 5 day Mixed = 26.4% of samples containing sapstain fungi.

For Export Trial I, Treatment 3 at the sampling point of Japan, only 12.7% of the samples contained sapstain fungi. This treatment had a high proportion *S. sapinea* isolated at the sampling point before shipment. The samples taken from the logs at the Port of Akita had *Trichoderma* species evident on the outer surfaces of most samples prior to processing in the laboratory in New Zealand. *Trichoderma* species dominated on the Media 4 selective agar plates therefore reducing the potential to isolate *S. sapinea*. *Trichoderma* species were inhibited on Media 6, allowing for successful isolations of *Ophiostoma* species.

The proportions of logs with *S. sapinea* and the total *Ophiostoma* species, species richness and dominance data for the different treatments and varying sampling periods are shown in Table 4.7 and Table 4.8 respectively. The effect of season was evident in the amount of sapstain fungi isolated when comparing Export Field Trial I (summer) and Export Field Trial II (winter). A total of 24% of the samples taken prior to anti-sapstain treatment contained *Ophiostoma* species compared with only 2% for the Export Trial II. More *Ophiostoma* species were isolated than *S. sapinea* in Trial I, with the exception of Treatment 2, Export Trial II. Treatment 1, with no anti-sapstain treatment, had more sapstain fungi than the anti-sapstain chemical treated logs. The timing of anti-sapstain treatment after harvesting was significant. When the logs were treated with anti-sapstain chemical within one day of harvesting there were less isolations of sapstain fungi.

At harvesting and pre-shipment *S. sapinea* was isolated (2 and 4 % respectively) more often than at the export destination (0.2%) at the ports in Japan for Export Trial I. For Export Trial II the isolations of *S. sapinea* remained constant. Isolations of *Ophiostoma* species, however, increased with each sampling time, from harvesting to pre-shipment in New Zealand to the most being isolated at the export destination in Japan for both Export Trials.

Table 4.7: The percentage (frequency) of *S. sapinea* and total *Ophiostoma* isolates, the richness and dominance of sapstain fungi isolated according to each treatment for Export Field Trial I and II.

	Treatment 1* No anti-sapstain		**Treatment 2* Anti-sapstain 4 days**		**Treatment 3* Anti-sapstain 4 days**		**Treatment 4* Anti-sapstain 1day**	
	Trial I	Trial II	Trial I	Trial II	Trial I	Trial II	Trial I	Trial II
S. sapinea	0.9 (6)	4.9 (45)	0.1 (1)	7.3 (67)	5 (33)	5.2 (48)	2.6 (17)	2.3 (21)
Total *Ophiostoma* isolates	62.7 (433)	10.0 (91)	23.8 (171)	6.6 (61)	11.5 (76)	6.3 (58)	21.4 (141)	6.5 (60)
Total sapstain isolates	63.6 (439)	14.9 (136)	23.9 (172)	13.9 (128)	16.5 (109)	11.5 (106)	23.9 (158)	8.8 (81)
Total number of wood pieces sampled	690	920	720	920	660	920	660	920
Richness	8	8	8	7	7	6	8	6
Dominant sapstain species [a]	*O. floccosum* *O. querci*	No	No	No	No	No	No	No

* Number in brackets indicates the frequency of sapstain fungi isolated.

[a] Dominant fungi according to Carmargo's index.

Table 4.8: The percentage (frequency) of *S. sapinea* and total *Ophiostoma* isolates, the richness and dominance of sapstain fungi isolated according to each sampling point for Export Field Trial I and II.

	Before AST*		**Before Shipment***		**Japan***		**New Zealand Parallel***	
	Trial I	Trial II	Trial I	Trial II	Trial I	Trial II	Trial I	Trial II
S. sapinea	2.2 (20)	1.6 (15)	3.9 (35)	1.6 (15)	0.2 (2)	2.7 (15)	No data	11.2 (103)
Total *Ophiostoma* isolates	24.7 (225)	1.5 (14)	27.2 (248)	3.5 (32)	38.2 (348)	18.4 (101)	No data	7.8 (72)
Total sapstain isolates	26.9 (245)	3.1 (29)	31.1 (283)	5.1 (47)	38.4 (350)	21.1 (116)	No data	19.0 (175)
Total number of wood pieces sampled	910	920	910	920	910	550	No data	920
Richness	7	5	8	5	8	7	No data	7
Dominant sapstain species [a]	No	No	No	No	*O. floccosum*	No	No data	No

* Number in brackets indicates the frequency of sapstain fungi isolated.

[a] Dominant fungi according to Carmargo's index.

The calculation of richness indicates the number of species within the community, however, it does not measure how many individuals are represented by each species in a community. For Export Trial I, richness did not vary according to treatment or sampling time A richness value of 8 was obtained for Treatments 1, 2 and 4 and a richness value of 7 for Treatment 3. Before anti-sapstain treatment, the richness value was 7 and increased to 8 for the sampling periods prior to shipment and at the Port of Akita, Japan. The dominant sapstain fungi as assessed by Carmargo's index were *O. floccosum* and *O.*

querci on the Treatment 1 (Table 4.7) and *O. floccosum* at the sampling point in Japan for Export Trial I (Table 4.8).

Values of richness for Export Trial II varied slightly with the different treatments. The highest richness value of 8 was recorded for Treatment 1, no anti-sapstain treatment. Treatment 2 and 3 (logs anti-sapstain treated within four days of harvesting) had richness values of 7 and 6, respectively, and Treatment 4 (logs anti-sapstain treated within one day of harvesting) had a richness value of 6 (Table 4.7). Richness increased from a value of 5, before anti-sapstain treatment and prior to shipment, to a richness value of 7 at the Port of Hamada in Japan (Table 4.8). There were no dominant fungi identified in Export Trial 2 using Carmargo's index (Table 4.7 and Table 4.8).

4.4.4 Summary

This is the first report of sapstain development and sapstain fungal colonisation on logs from harvesting to an export destination. Farrell *et al* (1998) studied the sapstain fungi of New Zealand and identified *S. sapinea* and thirteen *Ophiostoma* species as the major sapstain organisms. The fungi identified in these trials were species that were previously isolated in the survey of Farrell *et al* (1998) and described in the Chapter 3 of this thesis.

The sapstain fungi found in these trials differed in proportion to the survey data described in Chapter 3. *S. sapinea* was described as the cause of the majority of sapstain problems in New Zealand (Birch, 1936; Butcher, 1967; Farrell *et al*, 1998). The survey data (Chapter 3) showed that *S. sapinea* was dominant in summer, however, Export Trial I to Japan had higher amounts of *Ophiostoma* species than *S. sapinea.* In Export Trial II a total of 5% of samples taken contained *S. sapinea.* The isolation of very little *S. sapinea* in both trials was therefore a little surprising. The spores of *S. sapinea* are associated with cones and needles that are commonly found on the forest floor (Palmer *et al*, 1988). The inoculum density of *S. sapinea* is higher presumably, therefore, in the forest environment. As the logs during pre-shipment were stored in a processing yard the incidence of infection by spores of *S. sapinea* was potentially lower. More *Ophiostoma* species than *S. sapinea* were isolated in both trials. The sticky spores on the synemmatal and perithelial stalks of *Ophiostoma* species, are shown to be disseminated by wind and insect vectors, and are commonly found on logs and timber (Dowding, 1970). The insect vectors particularly the bark beetles (commonly *Hylastes* *ater* in New Zealand) are found both in logs and stumps (Reay *et al*, 2002). Insects and wind increase the spread of *Ophiostoma* species from logs at mill, processing plants and ports, therefore more inoculum potential for *Ophiostoma* species could occur at these sites.

The international spread of sapstain and vascular pathogens is strongly associated with movement of timber infested with an insect vector. Readily visible vectors such as bark beetles can be intercepted, but controlling microorganisms poses greater difficulties. A more important consideration in the movement of these logs is the risk that various pests will be carried from the country of origin into the importing country. No sapstain fungi considered serious pathogens (*Leptographium wagneri*, *Ophiostoma ulmi*, *Ophiostoma novo ulmi*, and members of the *Ceratocystis* family) were transported from New Zealand on either trial. Species of *Ophiostoma* are best known from the Northern Hemisphere and particularly Europe and North America. There are very few reports of *Ophiostoma* species in Japan and other Asian countries (Van der Westhuizen *et al*, 1995). Aoshima (1960) studied the wood-stain fungi of Japan and found *S. sapinea* and members of the Ophiostomataceae family, including the following *Ophiostoma* species, which are also isolated from New Zealand: *O. floccosum*, *O. piceae*, *O. pluriannulatum*, *O. stenocerus*, *O. piliferum*, *O. ips*, and *Leptographium* species. Both Export Trials showed that the majority of the sapstain fungi of New Zealand being exported to Japan are already present in that country. A comprehensive survey of the sapstain fungi of Japan, especially the fungi on imported wood, would be required to confidently say that a country such as New Zealand is exporting foreign sapstain organisms into Japan.

The severity of stain and the number of fungal isolations differed between the summer trial and the winter trial. The summer trial was more heavily stained and more fungi were isolated. Butcher (1967) when studying sapstain development on *P. radiata* posts above and below ground in New Zealand found stain in practically all posts from December to May. Butcher (1967) also compared the mean percentage staining with the monthly mean temperature and total rainfall and showed the incidence of sapstain was associated closely with these factors. For both trials the temperature, and rainfall or relative humidity was measured for the entire trial periods. The increase in temperature and humidity while on the ship did not make a significant difference to the amount of sapstain and fungal colonisation for the Export Field Trial II as indicated by the data.

There was very little stain prior to shipment and the logs were still relatively sapstain free on arrival at the export destination.

The time between harvesting and anti-sapstain treatment had a serious effect on the sapstain development in the summer trial. The amount of sapstain fungi that had infected the logs prior to anti-sapstain treatment in summer was high. Zeff (1999) and Cooper *et al* (2000) developed and verified a mathematical model (Sapstain Danger Index, SDI), which is used to calculate how soon after harvesting, a log should be treated in order to minimise sapstain development, by using parameters such as temperature, rainfall and relative humidity. Using this model, Zeff (1999) stated that the time between harvesting and treatment should be minimised and should not exceed 48 hours during the summer months. Results from this trial showed that anti-sapstain treatment within one day was more effective at controlling sapstain than when logs were treated four days after harvesting. Future work to correlate more closely export results with treatment time and with SDI could be used to optimise forest management parameters.

Sapstain fungi were isolated from logs treated with anti-sapstain chemicals. The anti-sapstain chemical treatments remain only on the logs surface (Morrell and Xiao, 1999) and could have killed off the surface spores and hyphae but not the sapstain fungi that had already successfully penetrated into the wood. Another possible reason could be the presence of chemical resistant sapstain fungi. Xiao and Kreber (1999) studied the effect of a chemical formulation IPBC/DDAC on spore germination and hyphal growth of *O. piceae*. Spore germination occurred within 24 hours on untreated wood and the majority of spores did not germinate on treated wood. However, in some spores, the process of germination was delayed in treated wood, but once it occurred, hyphae rapidly colonised the treated wood (Xiao and Kreber, 1999).

In conclusion from Export Trial I, nine species were isolated, the most common being *O. floccosum*, *O. querci* and *O. setosum*. Export Trial II had a different combination of nine sapstain species detected, the most common were *S. sapinea*, *O. querci*, *O. floccosum*, *O. setosum* and *O. piceae*. Logs arriving in Japan that were harvested in New Zealand summer had more stained logs and more severity of stain than logs harvested in winter. Results from this trial showed that anti-sapstain treatment within one day was more effective at controlling sapstain than when logs were treated four days post-harvest. This research showed the New Zealand sapstain fungi being exported on logs are present in Japan and no serious pathogens were detected.

5 Colonisation of sapstain fungi on *Pinus radiata*

5.1 Introduction

Information provided in Chapters 3 and 4 identified the sapstain species and their prevalence in New Zealand. During the ecological investigations on New Zealand species of sapstain fungi, a number of *Ophiostoma* species were encountered which were not well studied with regard to colonisation and stain formation on *P. radiata.* Colonisation data provides fundamental information and will assist in development of more effective strategies to control the staining fungi in the environment.

The growth and development of sapstain fungi is strongly influenced by the nutrient, moisture and oxygen content of the wood as well as the ambient temperature (Seifert, 1993). This is exemplified in New Zealand by the variation in the incidence of sapstain development between winter and summer as seen in Chapter 4. There was a lack of knowledge of the New Zealand isolates of sapstain fungi and their colonisation on *P. radiata*. The majority of the published studies on growth rates used agar plates, or lab sized wood specimens. Most of the previous colonisation work looked at stain development not fungal growth.

Gibbs (1993) stated that there was a lack of useful data on the rates of sapstain development in logs. He found that the majority of work on growth rates was on wood that had been altered by heat treatment or chemical sterilisation. He suggested that more work was needed on the growth of sapstain fungi in the host and on the extent that growth is influenced by environmental factors. Improved knowledge of the biology of these fungi may lead to better control strategies for sapstain in forest industry.

This chapter reports on the colonisation of sapstain fungi *in vitro* and on *P. radiata* in the field environment. The hypothesis, major aims and objectives of this chapter are firstly presented. A literature review is provided at the beginning of this chapter and introduces wood as a nutrient source for sapstain fungi. Literature on the environmental factors influencing the colonisation of sapstain fungi is given. This chapter provides methods and materials, results and a discussion on the colonisation of sapstain fungi in New Zealand with emphasis on the radial penetration and stain development on *P. radiata* in vitro and in the field environment.

5.2 Hypothesis, Aims and Objectives

The hypothesis of this chapter is that individual species of New Zealand sapstain fungi stain and colonise *P. radiata* differently. The aim of this chapter was to measure the intrinsic growth rates, stain development and colonisation of a number of New Zealand sapstain fungi, on synthetic media, on small specimens of *P. radiata* in the laboratory and in two field trials with *P. radiata* logs. Albino strains of *Ophiostoma* species were also assessed and will be described in Chapter 6.

The following objectives were addressed:

- To compare the intrinsic growth rates of four of the most commonly isolated New Zealand sapstain species *O. floccosum, L. procerum, S. sapinea* and *O. ips* on synthetic media *in vitro*.
- To investigate the radial penetration and colonisation of *O. floccosum, L. procerum, S. sapinea* and *O. ips* in the laboratory on unsterilised *P. radiata* specimens at three different temperatures.
- To assess the colonisation and stain development of nine New Zealand sapstain fungi *in vitro* on sterilised *P. radiata* specimens.
- To investigate the radial penetration and colonisation of *O. floccosum, L. procerum, S. sapinea* and *O. ips* in freshly cut *P. radiata* logs in the field during two seasons (New Zealand Summer and Winter).
- To ascertain the spatial distribution and colonisation of sapstain fungi in *P. radiata* using light microscopy.

5.3 Literature review

5.3.1 Colonisation of sapstain fungi

Saprophytic, pathogenic and endophytic fungi cause sapstain in wood. Saprophytic fungi are thought to be of greater economic significance, as this group invades logs after the tree is harvested (Seifert, 1993). The staining effect only becomes evident when conditions are favourable for fungal growth. Staining due to pathogenic and endophytic fungi is apparent when the tree is harvested and wood is discarded prior to processing (Seifert, 1993). Findley (1959) summarised much of the early work on the impact of environmental parameters on the colonisation of sapstain fungi. Much of the earlier work is clouded by the taxonomic debate and name changes within the

Ophiostomataceae Family. Seifert (1993) and Gibbs (1993) provide a more recent summary of the biology of sapstain with an emphasis *Ophiostoma* species.

Sapstain fungi are mainly isolated from sapwood as the name sapstain implies. It was observed though that the heartwood of *P. radiata* could be severely colonised by sapstain species (Thwaites and Farrell, 2000). Findley (1959a) also noted that sapstain within the heartwood of Douglas fir, Sitka spruce and pine could be occasionally found. Zheng *et al* (1995) discussed unsuccessful attempts to colonise the heartwood of lodgepole pine with *O. piceae*. Zheng *et al* (1995) also studied the influence of pH, moisture, nitrogen, fatty acids and mixed heartwood extractives on the growth of *O. piceae*. It was proposed that the toxicity of heartwood arose from the phenolic compounds present in lodgepole pine heartwood.

Much of the colonisation data presented on sapstain fungi include: the intrinsic growth rates of sapstain fungi on agar media; the microscopic colonisation of sapstain fungi in wood cells and the effect of different environmental parameters on colonisation.

The linear growth rates of fungi on malt extract agar for some of the major sapstain species on pine in Europe is shown in Table 5.1. However, most sapstain fungi grow more slowly through wood than on agar media (Uzunovic and Webber, 1998).

Table 5.1: Linear growth rate on malt agar at optimum temperature of some *Ophiostoma* species causing sapstain in pine and spruce (Source Gibbs, 1993).

Radial Growth Rate (mm/day)		
More than 10	Between 5 and 10	Less than 5
Ceratocystis coerulescens	*Ophiostoma clavatum*	*Ophiostoma brunneociliatum*
Leptographium lundbergii	*Ophiostoma ips*	*Ophiostoma canum*
Leptographium penicillatum	*Ophiostoma minus*	*Ophiostoma piceae*
Leptographium wingfieldii	*Ophiostoma polonicum*	*Ophiostoma piliferum*
	Ophiostoma huntii	*Leptographium procerum*
	Leptographium serpens	*Graphium spp.*

Gibbs (1993) stated that there was a lack of useful data on the rates of sapstain development in logs. Investigations into the growth of sapstain fungi in woody tissue almost invariably used wood blocks sterilised by heat, irradiation, or autoclaving. All these processes change the wood, sometimes making it too dry or depleting it of free soluble nutrients and substantially altering the natural and physio-chemical properties.

One of the few studies on penetration of sapstain fungi in wood is that of Lindgren (1942). Lindgren studied the growth of *O. piliferum* on blocks of *Pinus echinate* and found that longitudinal growth (4.5mm/day) was greater than radial or tangential (1.0 and 0.5mm/day respectively). The longitudinal growth in nutrient poor tracheid cells was compared with radial growth in the nutrient rich ray parenchyma cells. This study measured stain development in the wood rather than actual fungal penetration (Lindgren, 1942).

In the 1960s, Liese and Schmid undertook the most comprehensive investigations into the growth of *Ophiostoma* species in naturally stained *Pinus sylvestris* and *Picea abies* sapwood using microscopic analysis (quoted in Liese, 1970). Sapstain fungi are the primary or initial wood colonisers, entering the wood cell lumen, ray parenchyma cells, resin canals and the tracheids. Initial colonisation by sapstain fungi occurs with hyphae growing inside the parenchyma cells, where they obtain nutrients of carbohydrates, proteins and lipids (Liese, 1970). Fungal development within the rays is extensive before other non-living portions of the wood are colonised (Ballard *et al*, 1982).

Resin ducts lined by epithelial cells also provide a way into the sapwood (Ballard *et al*, 1982). Blanchette *et al* (1992) showed microscopically that the blue stained wood chips colonised by *O. piliferum*, had resin ducts free of resin and large concentrations of hyphae in the resin canals, surrounding epithelial cells and adjacent tracheids. Tangential sections through a resin duct showed hyphae throughout the resin canal growing within the residual resin.

In the late stages of sapstain colonisation the hyphae is widespread within the tracheids. The hyphae are also found growing on the cell wall surface of tracheids, however they do not reveal alteration of the cell wall surface (Liese, 1970). The hyphae pass from one tracheid to the next either by growing through pits or by direct penetration of the cell wall. The sapstain fungi open the parenchyma cells and rupture pit membranes but the tracheid cells remain intact (Blanchette *et al*, 1992). Liese (1970) believed that these fungi lack the enzyme system capable of degrading the cell wall constituents. The relative lack of lignification of the ray parenchyma cells of sapwood of pine species may be an apparent reason for the prolific growth and severe disruption of the ray cells. The heartwood sapwood boundary is more lignified, as is the heartwood itself, one reason why fungi may not be colonising the heartwood (Liese, 1970; Erikkson *et al*, 1990; Blanchette *et al*, 1992).

Sapstain fungi can produce hyphal appressorium at the point of hyphal attachment to the wood cell wall. The appressorium produce minute bore holes by the release of a fine penetration hypha about one fifth of the diameter of normal hypha that can penetrate the wall (transpressorium). On emergence into the lumen of an adjacent tracheid it reverts to its original diameter (Eaton and Hale, 1993; Gibbs, 1993: Seifert, 1993).

Schirp *et al* (1999) showed that at least three New Zealand isolates of *Ophiostoma* species and *S. sapinea* did not degrade the structural wood components of *P. radiata* using three independent quantitative methods. Some sapstain fungi were reported to exhibit cellulolytic, hemicellulolytic or pectinolytic activity in artificial media (Schirp *et al*, 1999; Schirp, 2001) but it appears that sapstain species lack the complete enzyme system to degrade cellulose, hemicellulose and lignin.

5.3.2 Factors influencing the growth of sapstain fungi

The nutrient, moisture and oxygen content of wood have been shown to influence the development of sapstain, as does ambient temperature, relative humidity and the wood type.

5.3.2.1 Nutrients

To utilise the available nutrients in wood, sapstain fungi must produce extracellular enzymes that hydrolyse the macromolecules in wood into assimilable nitrogen and carbon (Breuil, 1998). The majority of the nutritive substances in wood are present in the cytoplasm of parenchyma cells, lumen of tracheids and vessels and in the resin canals of the sapwood. Two types of substances are found; hydrophilic compounds (proteins, amino acids, starch and soluble sugars) and hydrophobic substances including wood extractives and resins.

Starch and lipids are the principle nutrition source in the storage tissue of wood (Zabel and Morrell, 1992). Starch is a readily available food source for sapstain fungi as it is present unmasked by any encrusting material in the cell. Sapstain fungi express amylases in order to use starch as a carbon source. King and Eggins (1973) tested 33 species of mould and sapstain fungi associated with colonisation of green timber for the degradation of amylose. All the species tested were considered amylotic. Umezurike (1969) showed that *B. theobromae* uses starch and other saccharides present in wood of *Bomba buonopozense* as initial substrates. Amylase activity was also detected in culture filtrates of *B. theobromae*. The amylase behaved like an inducible enzyme and was not detected in the cultures after exhaustion of starch.

Starch is generally recognised to be an important storage carbohydrate in wood, but soluble sugars can also constitute a major proportion of the total non-structural carbohydrate present (Cranswick *et al*, 1987).

Mathiesen-Käärik (1960) described the carbon utilisation of a number of sapstain fungi and found that there was a considerable variation in the utilisation of carbohydrates by individual sapstain fungi. The largest number of different carbon compounds were utilised by the primary pathogenic fungi, like *O. ulmi*. The secondary colonising sapstain fungi like *O. galeiformis* were found to utilise fewer compounds. All the sapstain fungi tested were able to readily utilise the hexose sugars including: glucose, fructose, mannose and galactose. The secondary colonising fungi were unable to utilise the di- and polysaccharides. Mathiesen-Käärik (1960) concluded that different ecological groups of sapstain fungi utilised different carbon sources. The secondary fungi, that invade the wood at a latter stage, usually entering logs already occupied by other organisms, are not able to utilise a great number of carbohydrates. These fungi may lack the enzyme systems to degrade the polysaccharides. Table 5.2 presents the data of Mathiesen-Käärik (1960) on the growth of *Ophiostoma* species found in New Zealand on different carbon sources.

Table 5.2: Growth of *Ophiostoma* species (found in New Zealand) on different carbon sources (source Mathiesen-Käärik, 1960).

Fungal species	*O. piceae*	*O. floccosum*	*O. galeiformis*
Glucose, fructose, mannose, galactose	+++	+++	+++
Cellobiose	+++	++	+++
Xylose	+++	+++	++
Maltose	+++	+++	++
Pectin	+++		+++
Glycerol	+++	+++	+++
Rhanmose	+++	+++	+++
Oleic acid	+++		+
Sorbitol	+++	++	+
Inositol	+++	++	+++
Arabinose	+++	+++	+++
Mannitol	+++	+++	+
Starch	++	+++	+
Sucrose	+++	+++	+
Sorbose	++	+++	++
Erthritol	++	++	+
Malic acid	+		+
Lactose	+	++	+
Raffinose	+	++	+
Inulin	++	+	+
Ribose	++	++	+
Cellulose	+	++	+

NB. +++ good growth; ++ fair growth; + poor growth; no sign – no experimental data.

In wood, the nitrogen available for fungal colonisation is low, usually between 0.01 and 0.1% of the dry weight (Merrill and Cowling, 1966). Most of the nitrogen is present as protein bodies found in the parenchyma cells, therefore, staining fungi would require extracellular enzymes to break down protein into assimilable amino acids to grow on wood. Staining fungi were shown to produce proteinases and aminopeptidases in protein-supplemented media and in wood (Breuil and Huang, 1994; Breuil *et al*, 1995). Immunogold labelling of a proteinase produced by a sapstain fungi revealed that the enzyme was secreted into the cell wall and released in a sheath surrounding the hyphae as the fungus grew in wood (Gharibian *et al*, 1996).

Lipids constitute most of the extractives in a tree and are present in amounts at least ten times greater than soluble sugars, and are a more concentrated form of energy. The total lipid content in the sapwood of most tree species is approximately 2% of the total dry weight (Gao *et al*, 1994). Triglycerides, which account for 40-50% of the total extractives, are easily hydrolysed by lipases into fatty acids and glycerol. Fatty acids and glycerol are used as a carbon source by the fungi (Mathiesen-Käärik, 1960; Gao *et al*, 1994; Zheng *et al*, 1995).

Wood extractives were shown to inhibit the growth of some fungi, however the *Ophiostoma* species tolerate and degrade resinous compounds (Blanchette *et al*, 1992). The degradation of resinous compounds by *Ophiostoma* species allows for the growth of other fungi that were sensitive to the resin (Blanchette *et al*, 1992). Blanchette *et al* (1992) showed that an albino strain of *O. piliferum* could decrease the total extractives and the esterified fatty acids of southern yellow pine wood chips. Gao *et al* (1994) showed that *O. piceae* was able to utilise the triglycerides, fatty and resin acids of *P. contorta*. Zink and Fengel (1990) showed that *O. piceae* was able to grow on fatty acids (C18:1 and 18:2) as a carbon source.

Recently, Martínez-Inigo *et al* (1999) confirmed that two other species of sapstain fungi, *Ophiostoma ainoae* and *Ceratocystis allantospora*, almost completely degraded triglycerides and long chain fatty acids in *P. sylvestris* sapwood. The fungal strains also reduced substantially the amounts of steryl esters and waxes in the wood. However, sterols and resin acids were not or were poorly removed by *Ophiostoma ainoae* and *Ceratocystis allantospora*.

In contrast, the resin acids of different wood species were reduced by the inoculation and growth of *Ophiostoma* species (Brush *et al*, 1994; Wang *et al*, 1995). Palustric acid and abietic acid were strongly reduced by the fungi tested, however, dehydroabietic acid and isopimaric acid were left as the dominant resin acids (Wang *et al*, 1995). The results suggested that biological pretreatment of chips was an efficient way of decreasing resin acids. In further studies on the degradation of the lipid content of wood, Leone and Breuil (1998) studied the degradation of aspen steryl esters and waxes and found that an albino strain of *O. piliferum* did not degrade these compounds efficiently.

The contrasting studies may be a reflection of the different requirement of the individual sapstain fungi or the different composition of the lipid component in the different wood species.

5.3.2.2 Moisture Content

Eaton and Hale (1993) described the moisture content of wood as the most important factor in determining the rate and extent of sapstain infestation in wood. The moisture content of sapwood in a vigorously growing living tree is too high to permit the growth of staining fungi, which are strongly aerobic (Findlay, 1959). For most *Ophiostoma* species it was suggested that an appreciable loss of moisture from freshly harvested wood is needed before fungal growth and penetration can occur (Gibbs, 1993). The loss of moisture may be induced by injuries or insect attack in standing trees or just seasoning in freshly harvested logs.

The limiting maximum moisture content for development of staining fungi can only be determined with regard to the density of the wood. The lower the density the more air and water the wood contains. The potential area of stain therefore increases during the drying of wood until fibre saturation is reached. In 1907, Münch observed a very slow penetration of *O. minus* into 5-10 cm discs cut from a 40-year-old *P. sylvestris* (quoted in Gibbs, 1993). However, a loss of only 10% of this moisture through air-drying was sufficient to allow complete penetration of the sapwood. Later studies by Lagerberg *et al* (1927), albeit on wood that was partially sterilised at 50°C, showed that *O. minus* was noticeably less inhibited by high moisture contents than were species such as *O. piliferum* and *L. lundbergii*. Lagerberg *et al* (1927) found that oxygen is the limiting factor of growth by sapstain fungi in wood with high moisture contents. Solheim (1991) suggested that rapid growth and ability to grow under oxygen-deficient conditions are important characteristics for primary sapwood invading fungi. Control of sapstain fungi is accomplished by sprinkling logs with water to inhibit fungal colonisation (Gibbs, 1993). The control works on the principle of keeping the moisture content of the wood high enough such that no fungi can colonise the wood.

The minimum moisture content for the growth of most sapstain fungi is about 27-28% (Findley, 1959). However, the minimum moisture content of about 24% was determined for stain development in *Pinus taeda* by *O. piliferum* (Lindgren, 1942; Björkman, 1946).

5.3.2.3 Oxygen

All filamentous fungi appear to be strictly aerobic (Berry, 1975). Although precise requirements for oxygen vary with the species and the growth conditions, most fungi grow as well at oxygen pressure of 20-40 mm Hg as at atmospheric pressure (160 mm Hg) (Berry, 1975). . Scheffer (1986) sought to establish the oxygen requirements of 48 wood-destroying Basidomycetes and six sapstain fungi, investigating i) growth of the fungi on nutrient agar under various partial pressures of oxygen; ii) capacity of the fungi to deplete oxygen when sealed in a closed chamber; iii) capacity of the fungi to survive when so confined. It was found that the six sapstain fungi survived one to six months without oxygen, exhibiting survival comparable to the majority of the Basidomycetes. *Ceratocystis minor*, *O. piliferum*, *Diplodia natalensis* and *Graphium rigidum* were able to survive between one and three months without oxygen, whereas *O. ips* survived up to six months without oxygen.

5.3.2.4 Temperature

Sapstain fungi grow on and stain wood optimally at temperatures between 22 and 30° C (Reynolds *et al*, 1972; Miller and Goodell, 1981). The minimum temperature for growth of sapstain fungi, depending on the species is around 0 –3° C and the maximum between 28 and 40°C (Käärik, 1980). Table 5.3 shows the cardinal (maximum, minimum and optimum) temperatures of *Ophiostoma* species found in New Zealand and summarised by Käärik (1980). Käärik (1980) also showed that *O. piliferum* was viable after 10 days at –21°C and that mycelium grows at –2.5°C after 35 days. Miller and Goodell (1981) looked at the ability of *O. piliferum* to grow and discolour at moderate and low temperatures and they found that the fastest rate of growth on malt extract agar was between 20 and 25°C. They also noted that severe staining of timber by *O. piliferum* occurred at temperatures of 3°C to 8°C when stored between one and two months (Miller and Goodell, 1981).

Table 5.3: Cardinal temperatures for growth of *Ophiostoma* species (source Käärik, 1980).

	Temperature (°C)		
Species	**Minimum for growth**	**Optimum for growth**	**Maximum for growth**
O. floccosum	<5	26	34
O. ips	<5	28-32	40
O. piceae	-2	23	35
O. piliferum	-3	26	34
O. pluriannulatum	4	28-29	35

5.3.2.5 Relative Humidity

Virtually no microorganisms are able to grow below the equilibrium relative humidity of 65% (Pasanen *et al*, 2000). Work by Mathiesen-Kaarik (1960) and Dowding (1969) found that spores of several wood staining fungi rapidly lost viability below 95% relative humidity and could only germinate in air at 100% humidity or in water. Payne *et al* (2000b) reported that spores and hyphae of *O. piceae* could develop and sporulate on Sitka spruce sapwood and on agar media at 93% relative humidity or higher.

This literature review concentrated on the growth aspects of sapstain fungi including growth conditions and colonisation on wood, and effects of moisture, oxygen, temperature and different nutrient requirements. These fungi colonise the parenchyma cells, resin ducts and tracheids of the wood, but they are not believed to damage the wood properties.

5.4 Materials and Methods

5.4.1 Fungal isolates

Isolates, representing four sapstain species were chosen for Colonisation Laboratory Experiments I and II and Colonisation Field Experiments I and I. The sapstain species, *S. sapinea, O. floccosum, O. ips*, and *L. procerum*, were selected as they represented distinct groups of sapstain fungi. *O. floccosum*, is a member of the *O. piceae* complex, *L. procerum* is a member of the *Leptographium* complex and *O. ips* has a *Pesotum* anamorph. These fungi were also dominant sapstain fungi in New Zealand for their specific groupings (Chapter 3). For Colonisation Laboratory Experiment I, three isolates of each species were assessed. For Colonisation Laboratory Experiment II and Colonisation Field Experiment I and II, one isolate of each species was assessed. For Colonisation Laboratory Experiment III, nine sapstain species were chosen for assessment. These fungi were selected as they were newly described from New Zealand in the ecological studies of Chapter 3 or not represented in the literature for stain development on *P. radiata*. Descriptions of the origin and date of isolation as well as the experiment that each strain was used in is provided Table 5.4.

Table 5.4: List of sapstain fungi used in each colonisation experiment with details of isolation.

Sapstain fungi	Isolate number	Experiment used	Origin	Date isolated
Botryosphaeria species	D19	Colonisation Laboratory III	Motueka Valley, South Island	10/10/96
Botryosphaeria species	D22	Colonisation Laboratory III	Hamilton, North Island	7/10/96
L. procerum	417	Colonisation Laboratory I	Riverhead Forest, North Island	6/05/97
L. procerum	446	Colonisation Laboratory I, III	Mahurangi Forest, North Island	22/05/97
L. procerum	1852	Colonisation Laboratory I, II; Colonisation Field I, II	Whitford Forest, North Island	29/01/98
L. truncatum	J696	Colonisation Laboratory III	Kinleith Forest, North Island	15/3/01
L. truncatum	J404	Colonisation Laboratory III	Kinleith Forest, North Island	15/3/01
O. coronata	431	Colonisation Laboratory III	Mahurangi Forest, North Island	28/05/97
O. coronata	868	Colonisation Laboratory III	Mahurangi Forest, North Island	20/06/97
O. floccosum	68	Colonisation Laboratory I, II; Colonisation Field I, II	Kinleith Forest, North Island	21/10/96
O. floccosum	148	Colonisation Laboratory I	Hanmer Springs, South Island	23/01/97
O. floccosum	J2004	Colonisation Laboratory I	Kinleith Forest	31/08/01
O. galeiformis	413	Colonisation Laboratory III	Riverhead Forest, North Island	5/05/97
O. galeiformis	832	Colonisation Laboratory III	Whitford Forest, North Island	9/07/97
O. huntii	474	Colonisation Laboratory III	Port of Tauranga, North Island	15/05/97
O. huntii	903	Colonisation Laboratory III	Tarawera Forest, North Island	25/06/97
O. ips	P36	Colonisation Laboratory I, II; Colonisation Field I, II	Kinleith Forest, North Island	12/01/00
O. ips	424	Colonisation Laboratory I	Northland, North Island	22/05/97
O. ips	1024	Colonisation Laboratory I	Mahurangi Forest, North Island	1/08/97
O. nigrocarpum	929	Colonisation Laboratory III	Tarawera Forest, North Island	26/06/97
O. querci	162 Mat b	Colonisation Laboratory III	Tasman Pulp and Paper, North Island	13/02/97
O. querci	1688	Colonisation Laboratory III	Whitford Forest, North Island	30/01/98
O. setosum	J1785	Colonisation Laboratory III	Kinleith Forest	9/05/01
O. setosum	670	Colonisation Laboratory III	Tauranga, North Island	14/05/97
O. stenocerus	930	Colonisation Laboratory III	Northland, North Island	1/07/97
S. sapinea	D4	Colonisation Laboratory I	Dome forest, North Island	26/09/96
S. sapinea	D33	Colonisation Laboratory I	Tauranga, North Island	8/12/96
S. sapinea	D35	Colonisation Laboratory I, II; Colonisation Field I, II	Kinleith Forest, North Island	13/12/96

5.4.2 Colonisation Laboratory Experiment I - Intrinsic growth rate assay

The intrinsic growth rates of selected isolates were measured on malt extract agar and malt yeast extract agar. Isolates were first grown on malt extract agar for approximately one week prior to establishment of the experiment. Discs 6mm in diameter were cut from the actively growing colony margins and placed at the centre of 90 mm plastic Petri dishes with three replicates per isolate, and incubated in darkness at the following temperatures 6°C, 15°C, 23°C and 30°C. Growth measurements at 6°C were suspended after the first cultures were assayed as the growth rates were too slow to get accurate measurements. Whether a culture was capable of growth at 6°C was then noted.

The first measurements of the colony diameters were taken after two days incubation at right angles. The second and subsequent measurements were made after one to four days depending on the speed of growth of the particular isolate. Growth measurements ceased after 30 days or when the colony was within 5 mm of the edge of the dish. The average intrinsic growth rate for each isolate was calculated as the mean radial increment per day, using the two measurements per plate, the daily measurements and the three replicate plates. Statistical analysis was performed as analysis of variance and Tukey's test for comparisons of means using Minitab 12 for Microsoft Windows.

5.4.3 Colonisation Laboratory Experiment II – Laboratory Radial Penetration and colonisation trial

Freshly harvested log bolts (200mm in length, 450mm in diameter) were selected *P. radiata* trees. The 25-year-old trees originated from Kinleith Forest, Central North Island, New Zealand. The discs were then sliced into specimens of 15 x 15 x 100mm (radial x tangential x longitudinal) from the sapwood and the orientations as shown in Figure 5.1. The specimens were stored at 5°C until required.

Each wood specimen was dipped in melted paraffin wax. The wax was allowed to solidify and the radial end that originated from the most outer edge of the log was sliced to remove the wax coating to allow for inoculation. Each specimen was placed in a separate clean resealable plastic bag. Inoculations of fungal spore suspensions occurred on the same day as wax dipping to minimize contamination. Unsterilised wood specimens were used in this experiment since these specimens mimic freshly harvested wood in the field.

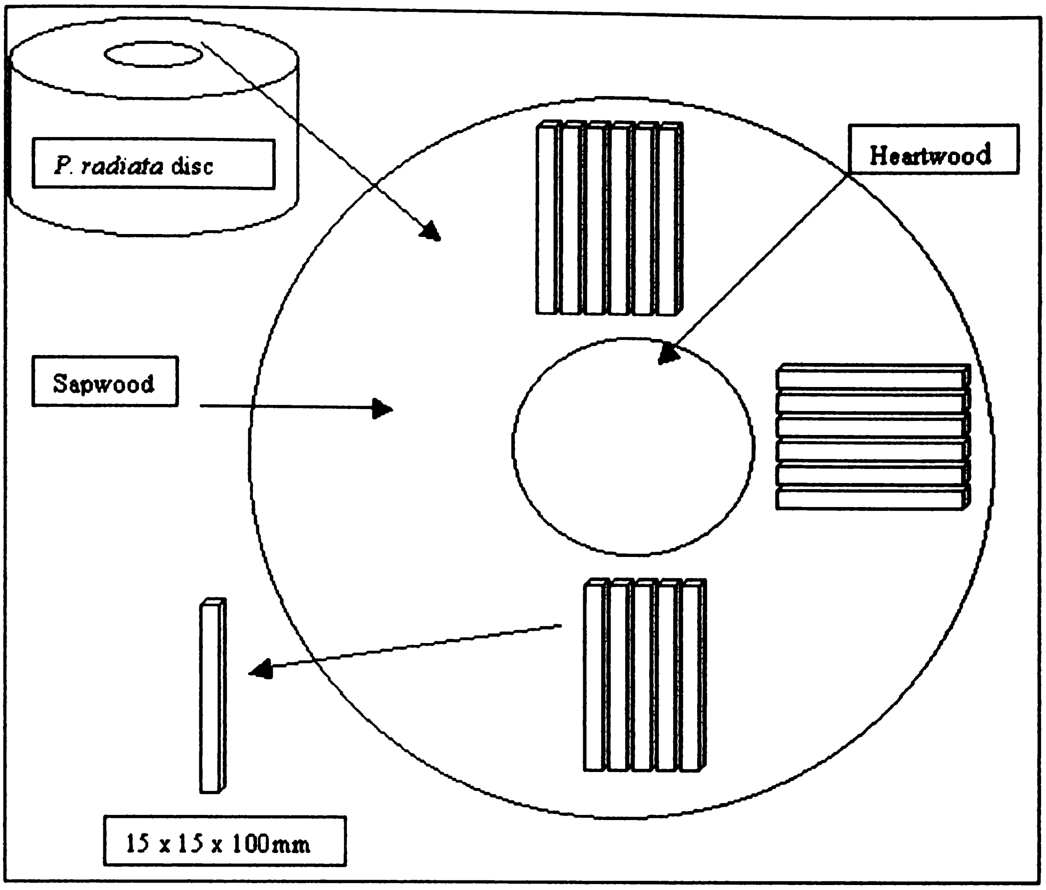


Figure 5.1: Diagram showing orientation of specimens used for the Colonisation Laboratory Experiment II.

The same fungal species (*S. sapinea, O. floccosum, O. ips, L. procerum*) used in the intrinsic growth rate assays were used in this experiment, but only one isolate of each fungus was selected. Albino isolates of *O. floccosum* F13 and F40 and *O. pluriannulatum* 3410 were also used but the results for these fungi will be shown in Chapter 6 (Section 6.5.4). Spore suspensions of approximately 1 X 10^6 spores/ml (or equivalent in dry weight (0.002g/ml) for *S. sapinea*) were used.

Nine specimens (replicates) per species and were incubated in darkness at the following temperatures: 15°C, 23°C and 30°C. After three days, one week or four weeks incubation time, three specimens per species, at all three temperatures were assessed for stain development and radial penetration.

The specimens were split in half (down the radial axis) with a sterile chisel and the stain development was measured with a ruler. The specimens were assessed for fungal radial penetration by slicing each specimen into the following ten increments from the outer most edge of sapwood to inner sapwood: 0-10, 10-20, 20-30, 30-40, 40-50, 50-60, 60-

70, 70-80, 80-90, 90-100mm. Each increment was further sliced and plated onto malt extract agar. The presence or absence of the inoculated fungi within each increment was noted and the average radial penetration of the three specimens per temperature, per time point was calculated.

5.4.4 Colonisation Laboratory Experiment III – Laboratory stain development and colonisation trial

Freshly harvested log bolts (200mm in length, 450mm in diameter) were selected from *P. radiata* trees. The 25-year-old trees originated from Kinleith Forest, Central North Island, New Zealand. The discs were then sliced into specimens of 20 x 20 x 40mm. Three specimens were placed in resealable plastic bags. Specimens were sterilised by gamma-irradiation (27.6kGy) at Mallickrodt Veterinary Ltd., Lower Hutt, New Zealand, prior to inoculation.

Two isolates of each of the following fungi were inoculated onto the wood specimens: *Botryosphaeria* species, *L. procerum*, *L. truncatum*, *O. huntii*, *O. galeiformis*, *O. setosum*, *O. querci*, and *O. coronata*. One isolate, each of *O. nigrocarpum* and *O. stenocerus* were inoculated. Three bags (each a replicate) containing three specimens were inoculated with each fungal isolate and three bags were inoculated with sterile water as a control. Two ml of fungal inoculum containing approximately 1 X 10^6 spores/ml (or equivalent in dry weight (0.002g/ml) for *Botryosphaeria* species) were added to each bag and thoroughly shaken. Samples and controls were incubated at 25°C for 6 weeks.

After six weeks incubation the specimens were cut in half with a sterile chisel and the internal surface of the specimen was assessed for stain development. Two independent assessors estimated the amount of visual sapstain coverage and the stain intensity value (using the criteria in Table 5.5) of the internal surface of each specimen. The average sapstain coverage and stain intensity value were determined from the average of both assessors and the average of the three specimens per bag.

Table 5.5: Stain intensity value scale.

Stain intensity Value	Description
0	No stain
1	Palest grey stain
2	Pale grey stain
3	Grey stain
4	Dark grey stain
5	Black stain

Small slivers were taken from the internal surface of each specimen and plated on to malt extract agar to ensure that the inoculated fungi had successfully colonised each specimen.

5.4.5 Colonisation Field Experiment I – Summer penetration field trial

The first trial was established at the University of Waikato, on the 20th of November 2000. The storage site was a secured grassed area within the grounds of the University with no public assesses (Figure 5.2). The University of Waikato was selected due to the ease of daily sampling, and assessment immediately after slicing. As this site was isolated from a forest environment, it was believed that less contamination from other sapstain fungi would affect the trial.

Figure 5.2: Site of Colonisation Field Trial I and II at The University of Waikato, Hamilton, New Zealand.

Logs were randomly selected and harvested the previous day from Kinleith Forest, Central North Island, New Zealand, debarked for the fungal treatments and with bark on for controls, and transported to the University. The logs were 1.2m in length with an average diameter of 31.8cm.

Three logs per treatment were inoculated as follows: *S. sapinea* (isolate D35); a mixture of *Ophiostoma* species (*L. procerum* isolate 1852, *O. ips* isolate P36, and *O. floccosum* isolate 148); and a mixture of two albino *O. floccosum* strains, F13 and F40. Three logs were treated with water that served as the control. The fungal species were inoculated at approximately 1 x 10^5 colony forming units per ml (or 0.002gm/ml of *S. sapinea* mycelium suspension) in a total of 4 litres of water and sprayed onto each set of three logs with a hand garden sprayer.

Each log was sampled every three days for a total of 30 days. A disc sample (50mm) was removed from one end of the log and discarded. A second disc sample (50mm) was subsequently sliced and this was assessed for stain development and radial penetration of the inoculated sapstain fungi. From each disc sample three wedges were taken (Figure 5.3). The moisture content of the first wedge was measured and calculated using the method described in Chapter 2 (Section 2.9). Visual sapstain development on the remaining two wedges was determined by measuring the radial stain development.

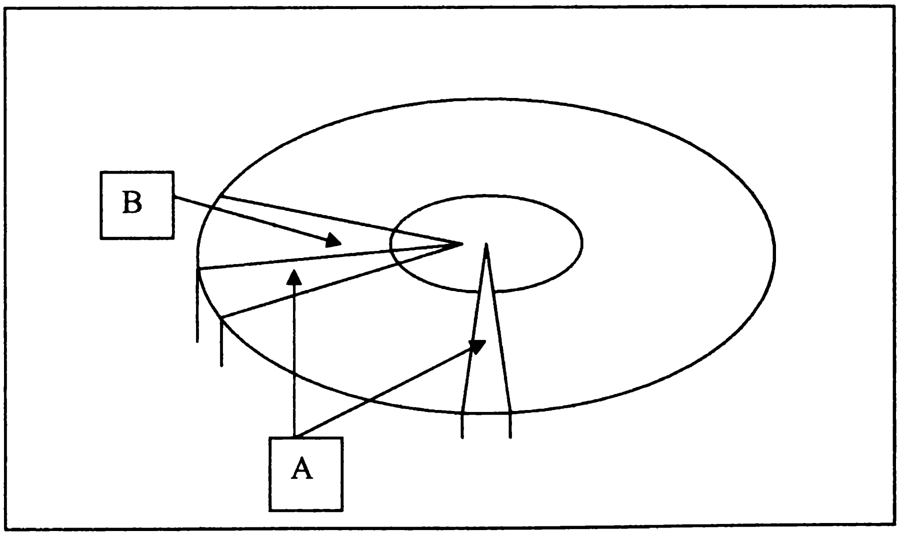


Figure 5.3: Cross section of a disc from *P. radiata* showing how samples were obtained for field trial growth rate assays. The position of the blocks taken for fungal isolation (A) and for moisture measurements (B) are indicated.

The two wedges were then used for culturing to measure the radial penetration of each inoculated fungus. Samples were taken in the following increments of each wedge with a chisel at Day 3: 0-5, 5-10, 10-15, 15-20, 20-25, 25-30, and greater than 30mm depths (Figure 5.4). Each increment sample was surface sterilised and slivers were then taken from the internal surface of each increment with a sterile scalpel. After Day 3, the increment sampling was increased due to the quick growth of *O. floccosum*, to the following depths: 0-5, 5-10, 10-15, 15-20, 20-25, 25-30, 30-40, 40-50, 50-60, and 60-70mm. After Day 18, increment sampling was again increased to the following depths: 0-10, 10-20, 20-30, 30-40, 40-50, 50-60, 60- 70, 70-80, 80-90, and 90-100mm. The scalpel samples were plated onto two selective media; Media 4 and Media 6 (Chapter 2 Section 2.2). All sapstain species were identified using morphological properties as described in Chapter 2 (Section 2.4).

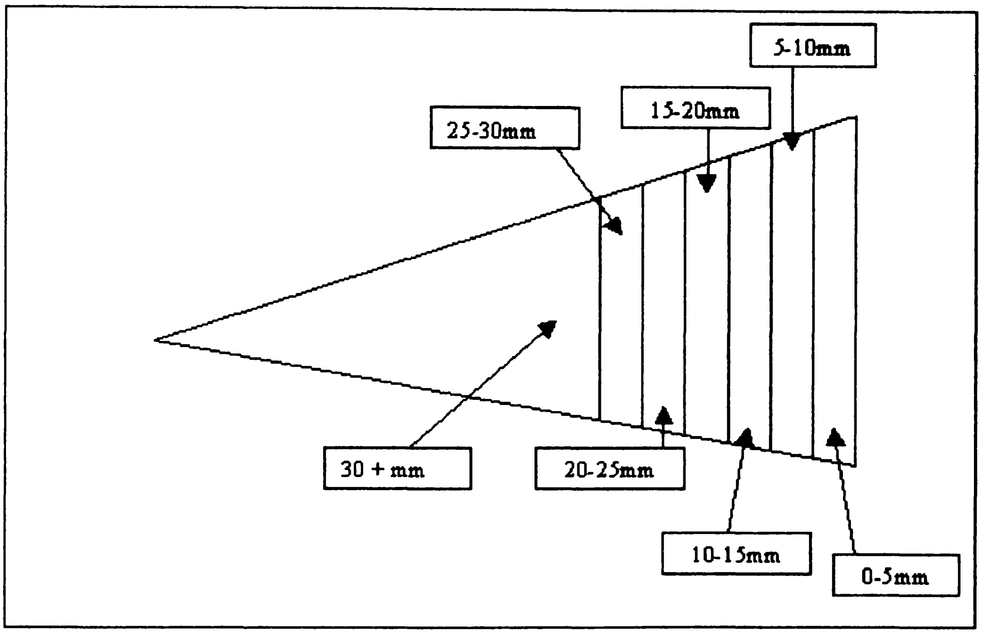


Figure 5.4: Increment sampling from a wedge taken for culturing to measure the radial penetration of sapstain fungi into the logs in Colonisation Field Experiment I at Day 3.

Weather data, both rainfall and temperature for the Hamilton area was also collected from a local weather station.

The resulting radial penetrating from culturing of fungi and visual stain data were reported as means and standard errors for the three logs per treatment. Statistical comparisons were performed using analysis of variance and Tukey's comparisons of means.

5.4.6 Colonisation Field Experiment II – Winter penetration field trial.

The first trial was established at The University of Waikato, on the 9th of July 2001 at the same site as Colonisation Field Experiment I. Logs were randomly selected and harvested the previous day from Kinleith Forest, Central North Island, New Zealand, debarked for the fungal treatments and with bark on for controls, and transported to the University as 200cm lengths.

Three logs per treatment were inoculated with the following criteria: *S. sapinea* (isolate D35); a mixture of *Ophiostoma* species (*L. procerum* isolate 1852, *O. ips* isolate P36, and *O. floccosum* isolate 148); and an albino *O. piuriannulatum* strain 3410. The albino *O. piuriannulatum* strain 3410 was chosen for this experiment as it is a successful biological control fungus developed in New Zealand for its ability to reduced sapstain see Chapter 6 (Section 6.5.6). Three logs were treated with water that served as the control. The fungal species were inoculated at approximately 1 x 10^5 colony forming units per ml (or 0.002gm/ml of *S. sapinea* mycelium suspension) in a total of 4 litres of water and sprayed onto each set of three logs with a hand garden sprayer.

During the first week the logs were sampled every day, then for the following weeks every 3 days until 30 days total as described for Colonisation Field Experiment I (Section 5.4.5). Samples were taken from the following increments of each wedge: 0-10, 10-20, 20-30, 30-40, 40-50, 50-60, 60-70 and greater than 70mm depths with a chisel. The trial was assessed and analysed as described for Colonisation Field Experiment I (Section 5.4.5).

5.4.7 Microscopical analysis of growth in wood

P. radiata sapwood samples inoculated with each of the following cultures independently and obtained from the Colonisation Laboratory Experiments II and III were examined microscopically after 4-5 weeks incubation: *O. ips* (isolate P36), *S. sapinea* (isolate D35), *Botryosphaeria* species (isolate D19) and *L. truncatum* (isolate J404). Radial and tangential sections of wood of approximately one or two wood cells in thickness were cut using a razor blade and placed on glass slides with water or stained with cotton blue in lactophenol. Cotton blue in lactophenol was used to enhance the contrast of fungal hyphae but it was subsequently noted that the fungal hyphae was easily recognisable without any staining. The prepared slides were analysed using an

Olympus BX40 light microscope and photographs were taken with a Nikon Coolpix 995.

5.5 Results and Discussion

It was the principle objective of this chapter to measure the intrinsic growth rates, stain development and colonisation of various New Zealand sapstain species on *P. radiata* in both the laboratory and the field. This results and discussion section presents data from three laboratory experiments and two field trials.

The sapstain species tested varied in their growth rates on synthetic media, and on laboratory specimens of *P. radiata* and on logs maintained in the field. There was variation in growth and colonisation at different temperatures and in different seasons between the species.

5.5.1 Colonisation Laboratory Experiment I - Intrinsic growth rate assay

All fungi tested grew at all temperatures and showed similar growth trends in both media. A summary of intrinsic growth rates for all sapstain fungi tested is shown in Table 5.6. *S. sapinea* was the fastest growing sapstain fungi of the species tested. The maximum growth was recorded at 30°C (9.4mm/day on malt yeast extract agar). *O. ips* was the next fastest with a maximum growth rate at 30°C (6.3mm/day on malt extract agar). Both *L. procerum* and *O. floccosum* had maximum growth rates (2.8 and 1.8mm/day respectively) at 23°C.

Table 5.6: Summary of the mean growth rates for the sapstain fungi on different media and at different temperatures.

	Mean growth rate (mm/day)[1]					
	15°C		**23°C**		**30°C**	
Sapstain species	**Yeast Malt**	**Malt**	**Yeast Malt**	**Malt**	**Yeast Malt**	**Malt**
L. procerum	1.8 ± 0.05	1.9 ± 0.07	2.7 ± 0.03	2.8 ± 0.10	0.4 ± 0.04	0.6 ± 0.04
O. floccosum	1.2 ± 0.01	1.3 ± 0.04	1.7 ± 0.02	1.8 ± 0.06	0.9 ± 0.07	1.0 ± 0.07
O. ips	3.0 ± 0.13	3.0 ± 0.12	4.4 ± 0.20	4.4 ± 0.20	6.0 ± 0.20	6.3 ± 0.20
S. sapinea	3.5 ± 0.17	3.6 ± 0.12	6.4 ± 0.21	6.3 ± 0.11	9.4 ± 0.15	9.0 ± 0.13

[1] Mean radial growth rate (mm) per 24 hours ± standard error.

O. floccosum grew slowly in both media with an average growth rate of 1.77mm/day at 23°C. There was a significant difference between growth rates for different isolates of *O. floccosum* (F = 10.39, P<0.000) and for growth rates on the malt versus yeast-malt agar (F= 19.29, P<0.000). Growth rates were highest for *O. floccosum* at 23°C compared to 15°C and 30°C (F =244.93, P<0.000). All strains of *O. floccosum* tested were also able to grow at 6°C but very slowly. Table 5.7 shows the growth rates for each strain and media at the three temperatures. Growth rates with the same letter following the standard error figure are not significantly different according Tukey's pairwise comparisons. *O. floccosum* generally grew faster in malt extract agar than malt yeast extract agar at all temperatures.

Table 5.7: Linear growth rate of *O. floccosum* at three different temperatures.

	Radial growth rate (mm/day)[1]		
***O. floccosum* isolate and media type**	***15°C***	***23°C***	***30°C***
68 YM	1.2 ± 0.01de	1.7 ± 0.02h	1.1 ± 0.01d
68 M	1.4 ± 0.01g	2.0 ± 0.03	0.9 ± 0.00c
148 YM	1.2 ± 0.02de	1.7 ± 0.03h	0.7 ± 0.00a
148 M	1.2 ± 0.03de	1.8 ± 0.02h	0.9 ± 0.02bc
J2004 YM	1.2 ± 0.03de	1.8 ± 0.03h	0.8 ± 0.02ab
J2004 M	1.4 ± 0.00fg	1.8 ± 0.01h	1.3 ± 0.04ef

[1] Mean radial growth rate (mm) per 24 hours ± standard error.

Values within the table with same letter do not differ significantly with a Tukey's analysis (P>0.05).

O. ips grew moderately fast in both malt and yeast malt agar with an average growth rate of 6.1mm/day at 30°C. There was no significant difference in growth rates for *O. ips* on malt or yeast-malt agar (F= 0.41, P=0.526). There was a statistical difference in the growth rate between different isolates of *O. ips* (F=30.73, P<0.000). The quickest growth was evident at 30°C and there was a significant difference between growth rates at the three temperatures (F = 370.03, P<0.000). Slow growth of this fungus was also evident at 6°C.

Table 5.8 shows the growth rates for each strain and media at the three temperatures. Growth rates with the same letter following the standard error figure are not significantly different according Tukey's pairwise comparisons. *O. ips* isolate number P36, was slower growing at all temperatures compared to the other isolates of *O. ips*. All the isolates originated from the North Island of New Zealand but isolates 1024 and 424 were from Northland and P36 was from Kinleith Forest in the Central North Island of New Zealand.

Table 5.8: Linear growth rate of *O. ips* at three different temperatures.

***O. ips* isolate and media type**	**Radial growth rate (mm/day)[1]**		
	15°C	***23°C***	***30°C***
P36 YM	2.6 ± 0.03a	3.7 ± 0.07cd	5.4 ± 0.03fg
P36 M	2.8 ± 0.09ab	3.7 ± 0.02c	5.8 ± 0.04gh
424 YM	3.4 ± 0.17bc	4.6 ± 0.20e	6.5 ± 0.31ij
424 M	2.7 ± 0.05ab	5.0 ± 0.1ef	7.1 ± 0.15j
1034 YM	3.0 ± 0.04ab	4.8 ± 0.05ef	6.2 ± 0.26ih
1034 M	3.4 ± 0.03bc	4.3 ± 0.10de	5.9 ± 0.06gh

[1] Mean radial growth rate (mm) per 24 hours ± standard error.

Values within the table with same letter do not differ significantly with a Tukey's analysis (P>0.05).

The fastest growth rates were recorded for *S. sapinea* of the four sapstain fungi tested for both media and at all temperatures. There was a significant difference in the growth rates on agar of *S. sapinea* according to temperature (F=689.56, P<0.000). Maximum growth was evident at 30°C. *S. sapinea* was also able to grow at 6°C but too slow to accurately obtain a daily growth rate. There was no difference between the growth rates on the different media (F=1.60, P=0.212) or between strains of *S. sapinea* (F=1.92, P=0.158). Table 5.9 shows the growth rates for each strain and media at the three temperatures. Growth rates with the same letter following the standard error figure are not significantly different according Tukey's pairwise comparisons.

Table 5.9: Linear growth rate of *S. sapinea* at three different temperatures.

***S. sapinea* isolate and media type**	**Radial growth rate (mm/day)[1]**		
	15°C	***23°C***	***30°C***
D4 YM	3.6 ± 0.15a	6.9 ± 0.22c	9.1 ± 0.10de
D4 M	3.9 ± 0.05a	6.5 ± 0.18bc	8.5 ± 0.13d
D33 YM	3.9 ± 0.06a	6.5 ± 0.15bc	9.5 ± 0.03e
D33 M	3.8 ± 0.10a	6.1 ± 0.13bc	9.1 ± 0.10de
D35 YM	3.1 ± 0.38a	5.7 ± 0.13b	9.8 ± 0.38e
D35 M	3.2 ± 0.09a	6.3 ± 0.24bc	9.3 ± 0.14de

[1] Mean radial growth rate (mm) per 24 hours ± standard error.

Values within a column with same letter so not differ significantly with a Tukey's analysis (P>0.05)

There was a statistical difference between growth rates at different temperatures for *L. procerum* (F=1064.5, P<0.000), maximal growth occurred at 23°C. There were also statistical differences in growth rates between strains (F=8.71, P=0.001) and between media (F=8.88, P=0.005). Table 5.10 shows the growth rates for each strain and media at the three temperatures. Growth rates with the same letter following the standard error figure are not significantly different according Tukey's pairwise comparisons. All isolates of *L. procerum* grew faster in malt extract agar than malt yeast extract agar at 15 and 23°C. There was no difference between growth rates of *L. procerum* in different media at 30°C.

Table 5.10: Linear growth rates of *L. procerum* at three different temperatures.

***L. procerum* isolate and media type**	**Radial growth rate (mm/day)[1]** ***15°C***	 ***23°C***	 ***30°C***
417 YM	1.9 ± 0.04ab	2.8 ± 0.04cd	0.4 ± 0.02fg
417 M	2.2 ± 0.03	3.1 ± 0.01	0.6 ± 0.02h
446 YM	1.6 ± 0.03a	2.6 ± 0.01ce	0.5 ± 0.01fg
446 M	1.9 ± 0.04b	2.8 ± 0.04d	0.6 ± 0.01gh
1688 YM	1.7 ± 0.01b	2.5 ± 0.12e	0.4 ± 0.00fi
1688 M	2.0 ± 0.00a	2.8 ± 0.04d	0.2 ± 0.00i

[1]Mean radial growth rate (mm) per 24 hours ± standard error.
Values within the table with same letter do not differ significantly with a Tukey's analysis ($P>0.05$).

The linear growth rates of sapstain fungi on synthetic media were well studied. The research present in this thesis on intrinsic growth rates on synthetic media was initiated to confirm the previous studies using New Zealand isolates of a variety of sapstain fungi and to compare with the results obtained on *P. radiata* in the laboratory and in the field.

Kay and Ah Chee (1999) studied the impacts of temperature on sapstain growth and development on artificial media using New Zealand isolates. The results present in this thesis endorse the findings of Kay and Ah Chee (1999). They found that *S. sapinea*, *O. ips*, *O. piliferum* and *O. piceae* had the most rapid rate of growth. *O. floccosum*, *O. piceae*, *O. piliferum*, *O. pluriannulatum* and *O. setosum* grew optimally at 20 to 25°C while *O. ips*, *S. sapinea* and *O. querci* grew optimally at greater than 25°C.

Kay *et al* (2002) measured the variation among New Zealand isolates of *S. sapinea*. It was found that there were significant differences among the growth rates on various media and between different isolates of *S. sapinea*. Chou (1987) found no differences in the growth rates of New Zealand isolates of *S. sapinea*. Variation has been widely documented for *S. sapinea* populations (Palmer *et al*, 1987; Swart *et al*, 1991).

Gibbs (1993) described the linear growth rates on malt extract agar of sapstain species. Of the species described that have been isolated in New Zealand *O. ips* and *O. huntii* had growth between 5 and 10 mm/day. *O. piliferum*, *L. procerum*, and *O. piceae* had linear growth rates less than 5mm/day. This data supports the results described by Gibbs (1993) for *L. procerum* and *O. ips*.

Variation between media was evident for some isolates of some species at different temperatures. For most of the species malt extract produced faster growth than malt extract agar. Benko and Highley (1990) studied the influence of different media on the growth of different wood inhabiting fungi including the sapstain fungi *Ceratocystis coerulescens*. The fungal growth (mm/day) of *C. coerulescens* was faster in malt extract agar than malt yeast extract agar at 27°C.

5.5.2 Colonisation Laboratory Experiment II – Laboratory Radial Penetration and colonisation trial.

Testing colonisation and radial penetration of fungi on wood is more desirable and accurate than on synthetic media. One isolate of each species of the fungi used in the Colonisation Laboratory Experiment I was selected for further analysis on *P. radiata* wood specimens.

Analysis of only the radial penetration of the fungi was chosen for this study, as the majority of fungal stain development evident in the Export Field Trial I was in a wedge shape projecting from the outer cambium in towards the heartwood (Chapter 4, Section 4.4.2). It is well documented that the sapstain fungi initially colonise the ray cells and grow radially toward the centre of the log (Ballard *et al*, 1984). Radial penetration was measured in this experiment in two ways. Firstly, the visual sapstain present on the inner surface of the wood was measured. Solheim (1992) noted that during the early stages of fungal colonisation the sapstain fungi are colourless, so the extent of visual sapstain is not necessarily a good indication of the progress of fungal penetration. The second method identified the radial penetration of colourless hyphae by culturing the fungi growing in small increment steps down the radial axis of the wood specimen.

5.5.2.1 Stain Development

The development of stain on wood specimens of the internal surface of *P. radiata* after 30 days incubation is shown in Figure 5.5. No stain was visible on the specimens sampled after three and seven day's incubation with any sapstain fungi. The visual appearance of penetrating stain was more advanced at 30°C for *O. ips* (Figure 5.6), *S. sapinea* and *O. floccosum*. No stain was visible on the *O. floccosum* wild type stain and the albino *O. floccosum* stains F13 and F40 at 15 and 23°C. No visible stain was evident on the specimens inoculated with *S. sapinea* at 15°C.

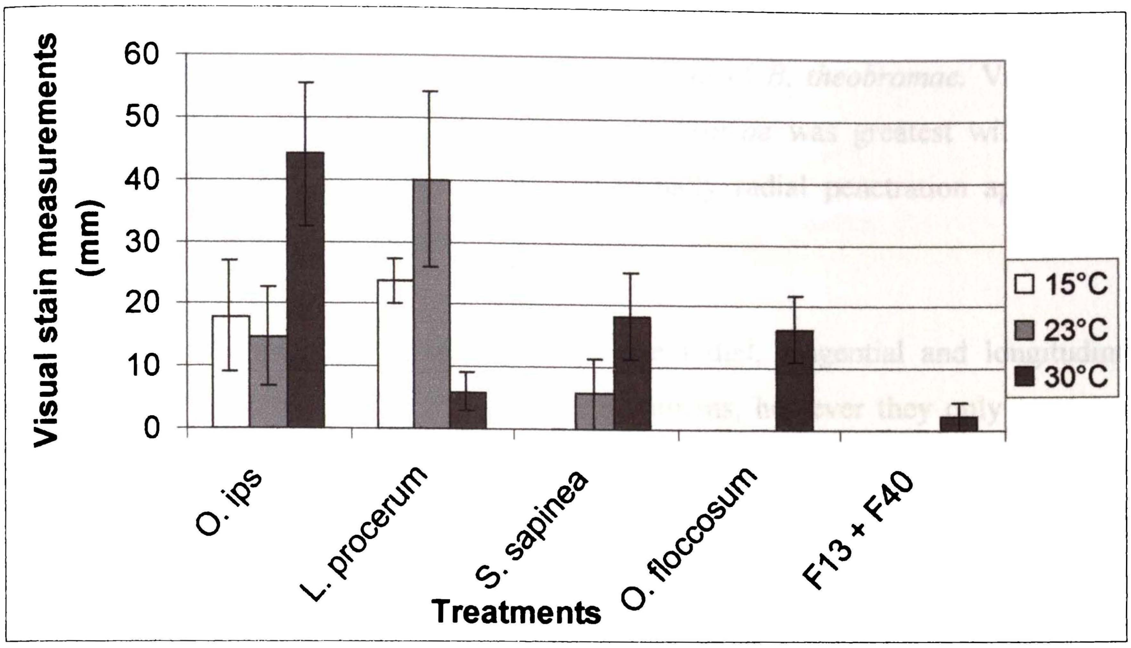


Figure 5.5: Visual stain development on laboratory specimens after 30 days incubation at various temperatures.

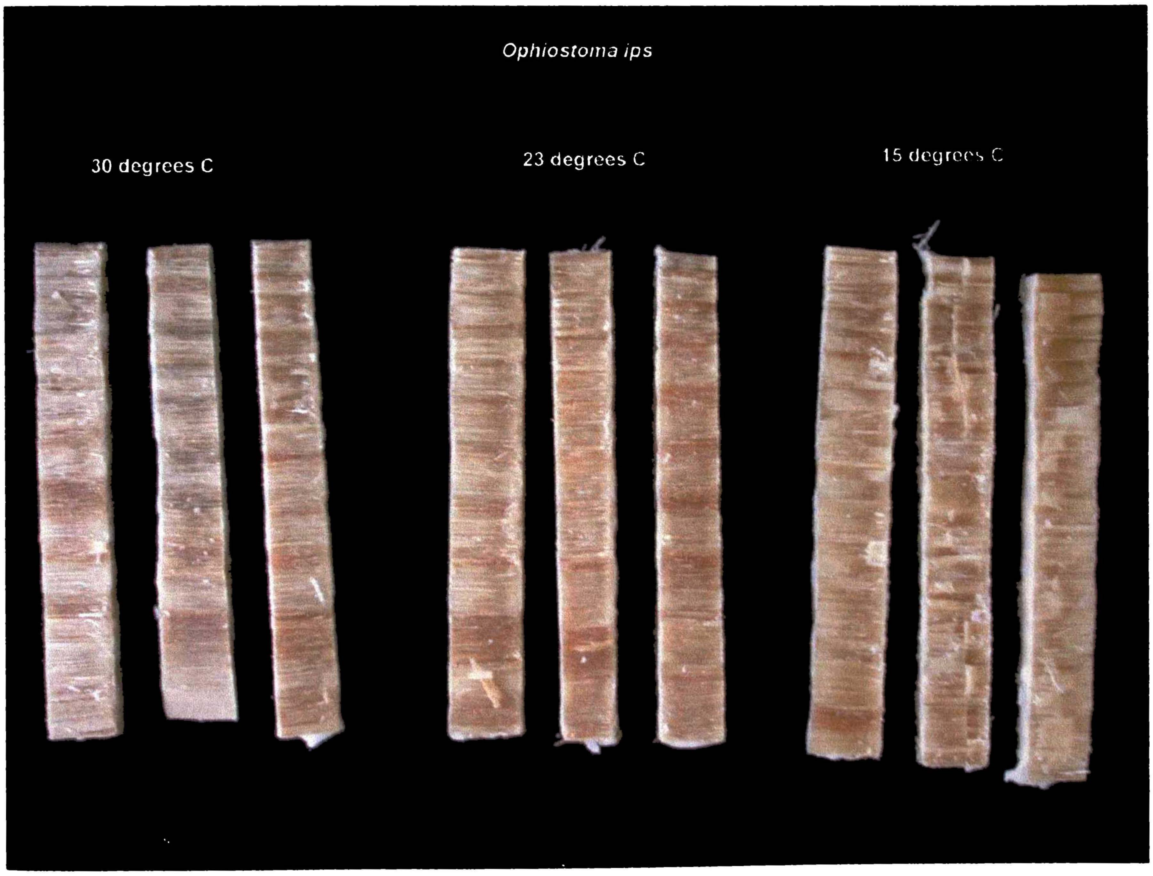


Figure 5.6: Visual sapstain on laboratory specimens after 30 days incubation with the *O. ips*.

Tabirih and Seehann (1984) studied the radially, tangential, and axial penetration of *Botryodiplodia theobromae* Pat. in specimens (2 X 2 X 10 cm) of a West African wood species, *Triplochiton scleroxylon* (Abachi). They found that the anatomical direction of this wood species greatly influences the visual spread of *B. theobromae*. Visual axial stain development of the sapstain species *B. theobromae* was greatest with 100mm penetration after 4 weeks. They found that visually radial penetration appeared at surprisingly low rates for the fungus.

Uzonovic and Webber (1998) also measured the radial, tangential and longitudinal penetration of sapstain fungi in *P. sylvestris* specimens, however they only measured visual stain development. They found that longitudinal growth of the fungi was faster than radial and tangential. In their experiment, radial growth of *S. sapinea* ranged between 0.6 to 2.3mm/day and found that *O. piceae* had no radial penetration (Uzonovic and Webber, 1998).

The results presented in this thesis research suggested that for the sapstain fungi tested visual sapstain development in the laboratory depended on the temperature of incubation. Stain development was more prominent at 30°C for the majority of fungi tested with the exception of *L. procerum*. In this experiment only the radial penetration was measured. The results of Uzonovic and Webber (1998), where no visual stain penetration was evident for *O. piceae* may be a reflection of the temperature (20°C) at which this experiment was conducted.

The fungal penetration in each specimen was measured by culturing the fungi from 10mm increment samples along the entire 100mm length. A comparison of the stain results and the fungal penetration results are compared and displayed in Section 5.5.2.3.

5.5.2.2 Fungal Penetration

The radial penetration of *O. floccosum* (strain 68) on *P. radiata* wood specimens at three temperatures is shown in Figure 5.7. *O. floccosum* grew to 10mm after three days at all temperatures. After seven days the fastest average growth of *O. floccosum* was 35mm at 30°C. After 30 days the maximum average growth of *O. floccosum* on the laboratory specimens was 50mm at 23°C. There were no statistical differences between any of the means at any temperature or any time point for *O. floccosum*.

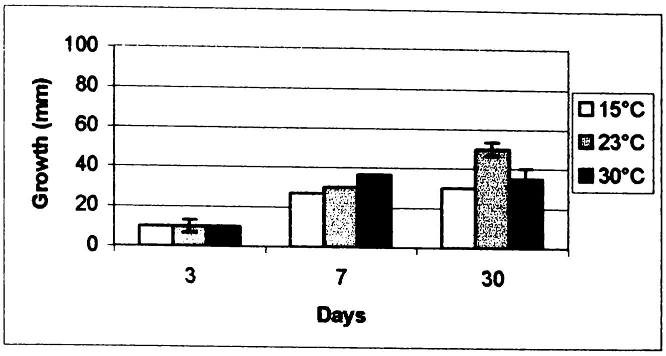


Figure 5.7: Growth of *O. floccosum* on laboratory specimens after various inoculation periods at various temperatures.

The radial penetration of *O. ips* (strain P36) on *P. radiata* wood specimens at three temperatures is shown in Figure 5.8. *O. ips* colonised the wood specimens between 10 and 15mm after three days for all temperatures. There was slightly more radial growth after seven days to 20mm. After 30 days the maximum average growth of *O. ips* was 75mm at 30°C, 65mm at 23°C and 35mm at 15°C. There was no statistical difference between the means of growth after 30 days inoculation for 23°C and 30°C. However, *O. ips* was statistically slower at penetrating the wood at 15°C after 30 days incubation time compared to both 23°C and 30°C.

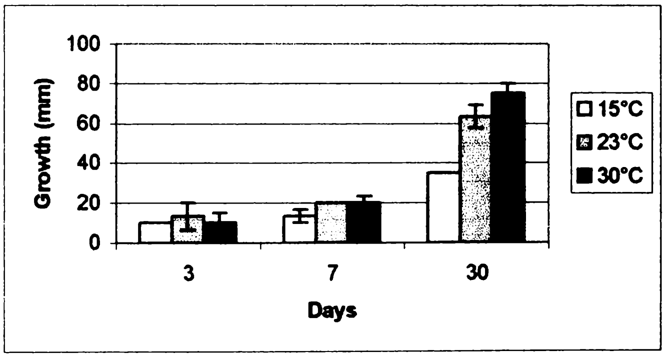


Figure 5.8: Growth of *O. ips* on laboratory specimens after various inoculation periods at various temperatures.

The radial penetration of *L. procerum* (strain 1852) on *P. radiata* wood specimens at three temperatures is shown in Figure 5.9. The fastest radial growth of *L. procerum* in the wood specimens was at 23°C. *L. procerum* showed a statistical difference in growth between all three temperatures after 30 days inoculation according to Tukey's analysis of means (P=0.05). *L. procerum* penetrated further into the wood with an average penetration of 75mm at 23°C than at the other temperatures (10mm at 30°C and 35mm at 15°C).

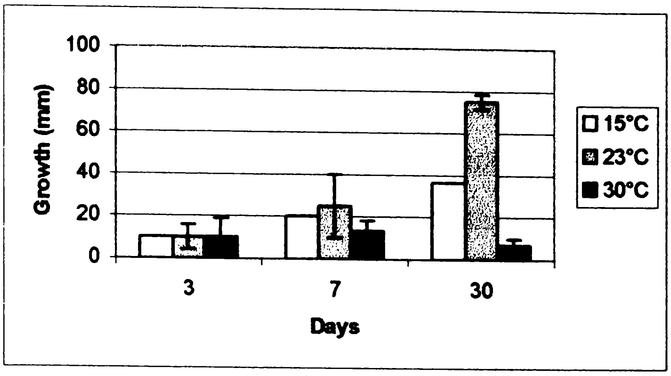


Figure 5.9: Growth of *L. procerum* on laboratory specimens after various inoculation periods at various temperatures.

The radial penetration of *S. sapinea* (strain D35) on *P. radiata* wood specimens at three temperatures is shown in Figure 5.10. *S. sapinea* grew to an average of 10mm after three days at 15 and 30°C and to 13.3mm at 23°C. After seven days the fastest average growth of *S. sapinea* was 17mm at 23°C. After 30 days the maximum average growth of *S. sapinea* on the laboratory specimens was 65mm at 23°C and 60mm at 30°C. *S. sapinea* was able to remain viable at 15°C but unable to penetrate into the wood. There was no statistical difference between the growth rates of *S. sapinea* at 23°C or 30°C at any time point.

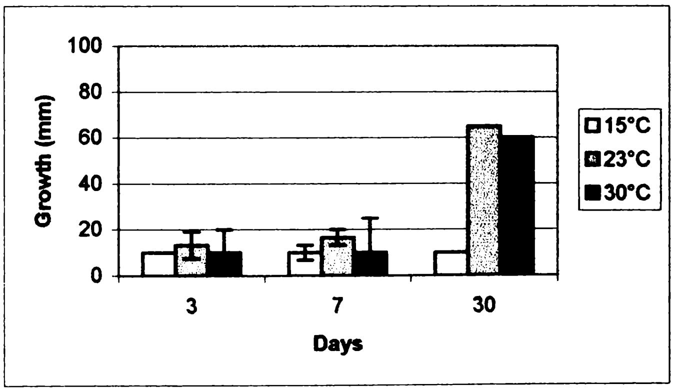


Figure 5.10: Growth of *S. sapinea* on laboratory specimens after various inoculation periods at various temperatures.

S. sapinea, *O. ips* and *L. procerum* grew more rapidly on the specimens than *O. floccosum* after 30 days incubation at 23°C. The data for the albino *O. floccosum* strains F40 and F13 and *O. piriannulatum* strain 3410 is presented in Chapter 6 (Section 6.5.4).

5.5.2.3 Comparison of stain development and fungal penetration

A comparison between the visual stain development and the fungal penetration from culturing at the three temperatures after 30 days incubation is shown in Table 5.11. Fungal penetration was always greater than the visual stain development. Solheim (1992) found that sapstain was closely correlated with fungal penetration, with visual sapstain observed between 2 to 3 mm behind the leading edge of fungal penetration.

Temperature appears to affect the melanisation of the hyphae in the sapstain fungi *O. floccosum* as this fungus was successful at penetrating radially but no stain developed at 15°C and 23°C. *S. sapinea* was unable to produce visible stain at 15°C. Lindgren (1942) studied the impact of temperature on wood staining fungi and found that *O. ips* grew optimally between 25°C and 30°C. The time to appearance and rate of hyphal melanisation was observed to vary with the different isolates, and the progression of melanisation was more rapid as temperatures approached optimal for growth of the isolate.

Table 5.11: Comparison of the visual stain data and the fungal penetration data for Colonisation Laboratory experiment II after 30 days incubation.

	15°C		23°C		30°C	
Sapstain fungi	**Visual stain (mm)**	**Fungal penetration (mm)**	**Visual stain (mm)**	**Fungal penetration (mm)**	**Visual stain (mm)**	**Fungal penetration (mm)**
O. ips	18	35	15	63	44	75
L. procerum	24	37	40	75	6	7
S. sapinea	0	10	6	65	18	60
O. floccosum	0	30	0	50	16	35

5.5.3 Colonisation Laboratory Experiment III – Laboratory stain development and colonisation trial

For many of the *Ophiostoma* species isolated in the survey of sapstain fungi in New Zealand it was unclear whether they imparted a sapstain to *P. radiata*. *P. radiata* laboratory size specimens were inoculated with various *Ophiostoma* species and two isolates of *Botryosphaeria* species. After six weeks incubation at room temperature (23°\C) the samples were assessed for stain development. Samples were also taken from the wood specimens for culturing to confirm the isolate identity. Only one isolate of each *O. nigrocarpum* and *O. stenocerus* were used in this experiment as the New Zealand culture collection of sapstain fungi from the survey contained one viable culture of each of these species.

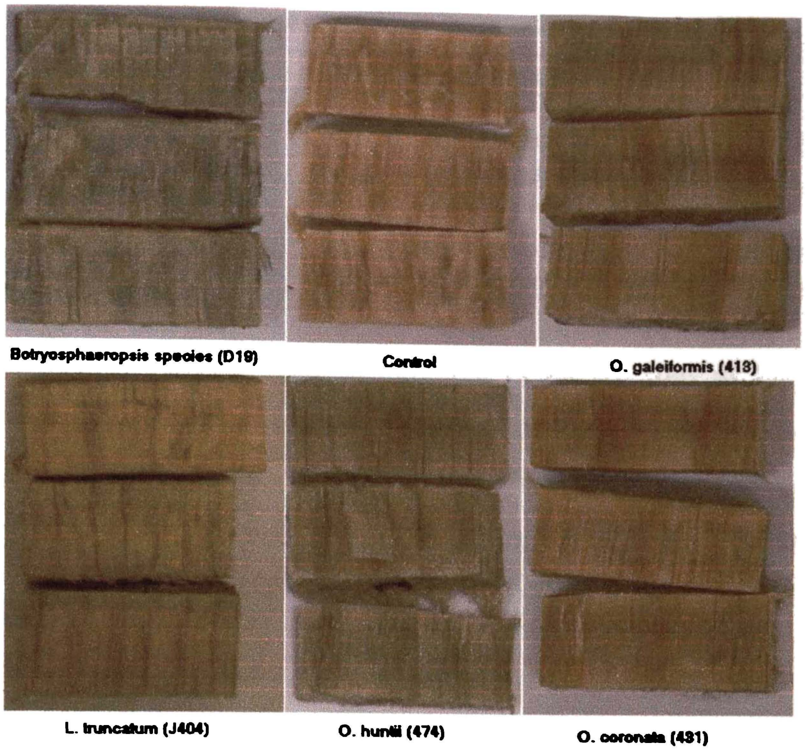
The mean stain intensity and mean percent coverage of stain on the *P. radiata* specimens is shown in Table 5.12 and pictures of the stain development of a number of stains are shown in Figure 5.11. The two isolates of *Botryosphaeria* species were the only fungi to extensively stain the specimens a dark grey to black stain. *L. procerum*, *L. truncatum*, *O. galeiformis* and *O. huntii* stained the wood a light grey colour. *O. setosum*, *O. nigrocarpum* and *O. stenocerus* had a very small amount of stain evident on a few specimens. The majority of the wood was clean. *O. coronata* and *O. querci* did not stain the wood in this experiment.

Table 5.12: Mean stain intensity and mean percent coverage of stain for *P. radiata* specimens inoculated with various sapstain species after six weeks incubation.

Species	Isolate Number	Mean stain intensity value*	Mean percent coverage of stain*
Control		0	0
Botryosphaeria species	D19	4.3 ± 0.06	93 ± 3.8
Botryosphaeria species	D22	4.7 ± 0.15	83 ± 4.5
L. procerum	446	0.5 ± 0.10	24 ± 9.4
L. truncatum	J404	0.9 ± 0.45	41 ± 20.3
L. truncatum	J696	1.4 ± 0.53	56 ± 17.5
O. coronata	431	0	0
O. galeiformis	413	1.4 ± 0.53	47 ± 3.9
O. galeiformis	832	1.0 ± 0.10	54 ± 12.2
O. huntii	474	1.3 ± 0.73	57 ± 28.9
O. querci	162	0	0
O. querci	1688	0	0
O. setosum	670	0.2 ± 0.2	9 ± 8.9
O. setosum	J1785	0.3 ± 0.2	19 ± 17.0
O. nigrocarpum	929	0.1 ± 0.05	5 ± 3.5
O. stenocerus	930	0.2 ± 0.1	5 ± 5

*the standard error is displayed after ±.

O. galeiformis, was found by Hutchison and Reid (1988b) to stain *P. radiata* a yellow to brown colour. The results in this thesis confirm the observations of Hutchison and Reid (1988a) that *O. coronata* does not stain *P. radiata*. *O. setosum* and *O. querci* were not significant stainers of *P. radiata*, as these species are closely related to *O. floccosum* it could be that these fungi would stain the wood when exposed to higher temperatures as was shown in Section 5.5.2.



Botryosphaeropsis species (D19)
Control
O. galeiformis (418)
L. truncatum (J404)
O. huntii (474)
O. coronata (431)

Figure 5.11: Wood specimens showing various stain intensity and coverage of stain for various sapstain species and a control on *P. radiata.*

5.5.4 Colonisation Field Experiment I – Summer penetration field trial

There is a general lack of data available on the growth of sapstain fungi in wood especially under natural field conditions. With these points in mind, the two field trials were established to measure colonisation of sapstain fungi on standard mature harvested unsterilised logs in the field.

A total of 39 samples were collected from the logs before spraying with treatments. Table 5.13 shows the sapstain species that were isolated from these chisel samples. A total of 12 isolates of *S. sapinea* were obtained from various positions on the logs including knots and wood samples from the top and end faces of the logs. The other sapstain fungi included *O. ips*, *O. setosum*, unidentified *Ophiostoma* species with black hyphae (potentially *O. huntii* or *O. ips*) and an unidentified *Ophiostoma* species with black synnema (potentially *O. querci* or *O. piceae*). As these logs were freshly harvested and transported within one day to The University of Waikato the source of contamination of these logs could be in transportation, at harvesting or these fungi were endophytes. Other types of fungi and yeasts isolated from the samples included *Trichoderma* species, *Fusarium* species, *Verticillium* species, *Alternaria* species, *Epicoccum* species and other unidentified fungi.

Table 5.13: Sapstain species isolated from logs prior to treatment inoculation.

Treatment	Sapstain species isolated
Albino *O. floccosum* strains (F13 and F40)	*S. sapinea*, *Ophiostoma* sp. with black hyphae
Control	*S. sapinea*, *O. setosum*
S. sapinea	*S. sapinea*, *O. ips*
Mixed *Ophiostoma* sp.	*S. sapinea*, *Ophiostoma* sp. with black hyphae, *Ophiostoma* sp. with black synnema, *O. ips*

Figure 5.12 shows the average moisture content of all the logs as the month of the summer trial progressed and the rainfall for the trial period. There was a rapid decline in moisture of the logs between Day 3 and Day 7. The relationship between the weather conditions for the same period was considered when looking at the moisture contents of the logs. An increase in moisture content on Day 24 could be related to heavy rainfall on Day 19, Day 20 and Day 22.

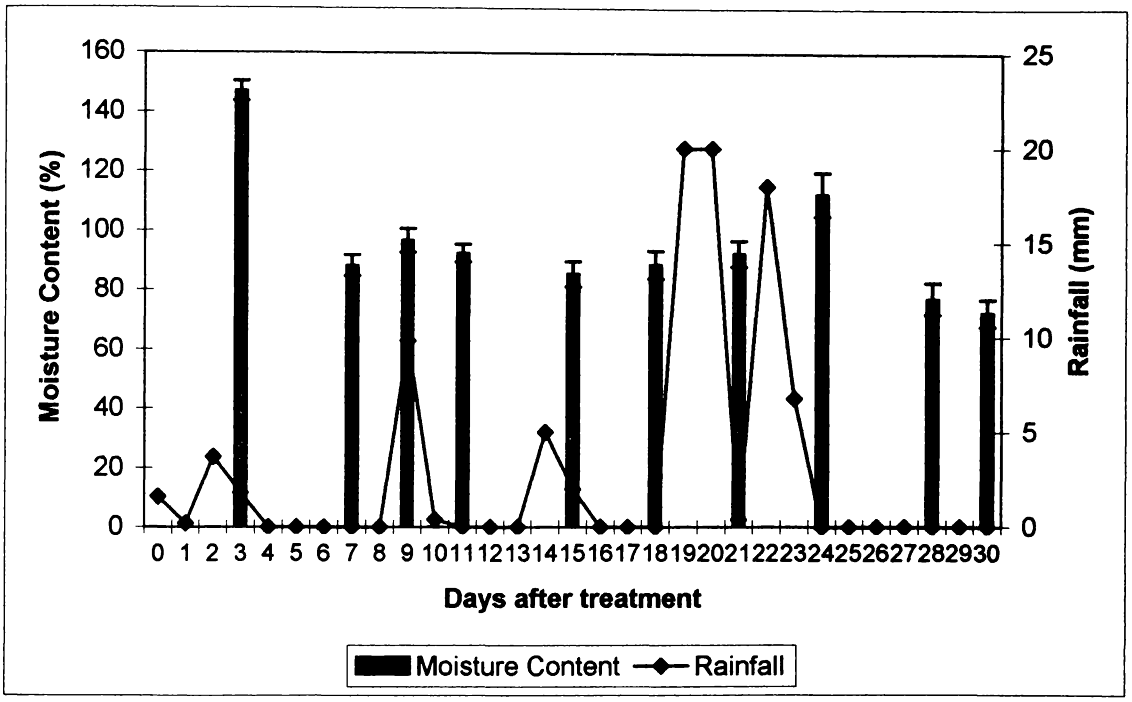


Figure 5.12: The average moisture content of logs in Colonisation Field Experiment I over a one-month period.

Figure 5.13 shows the average visual stain for each treatment for Colonisation Field Experiment I. The first visual evidence of sapstain on the logs was on Day 11 on the *S. sapinea* treatment with an average of 3.3mm stain. On Day 15 all of the treatments showed some visual sapstain. Visual sapstain on all treatments increased over time.

Figure 5.14 shows the increase in sapstain development on the log number D2 treated with *S. sapinea* from Day 15 to Day 30. Sapstain appeared on the cross-section of the disc around the majority of the circumference extending radially towards the heartwood (Figure 5.14 picture D). There was a statistical difference in the amount of stain developed between days (F=56.8, P<0.000). A large variation in the stain development between logs was evident for each treatment. Statistically there was a difference in stain development on the logs inoculated with different sapstain fungi and the control (F=9.41, P<0.000). These results showed that a period of 15 days of log storage during summer was enough for significant stain to develop in this trial.

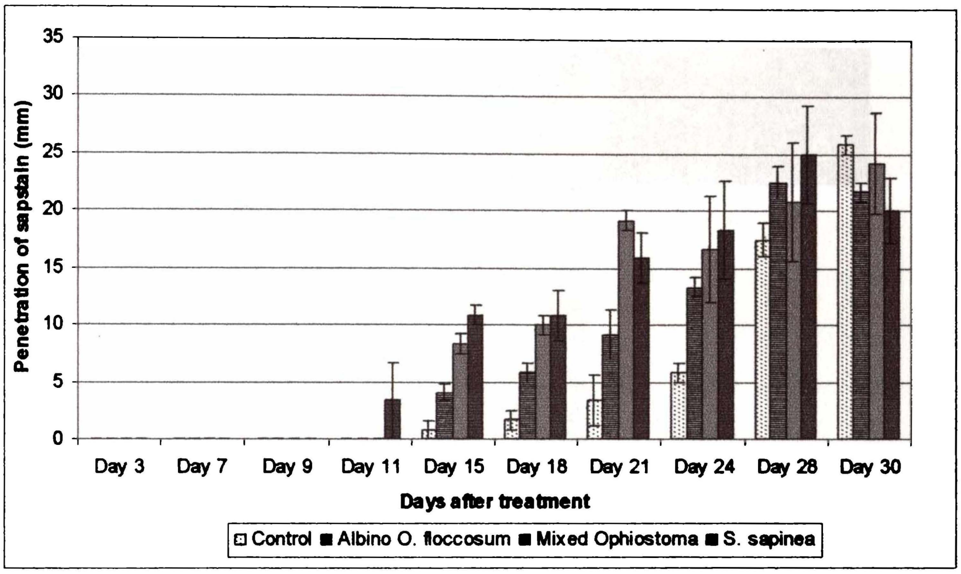


Figure 5.13: Visual sapstain present on each treatment over the month period for Colonisation Field Experiment I.

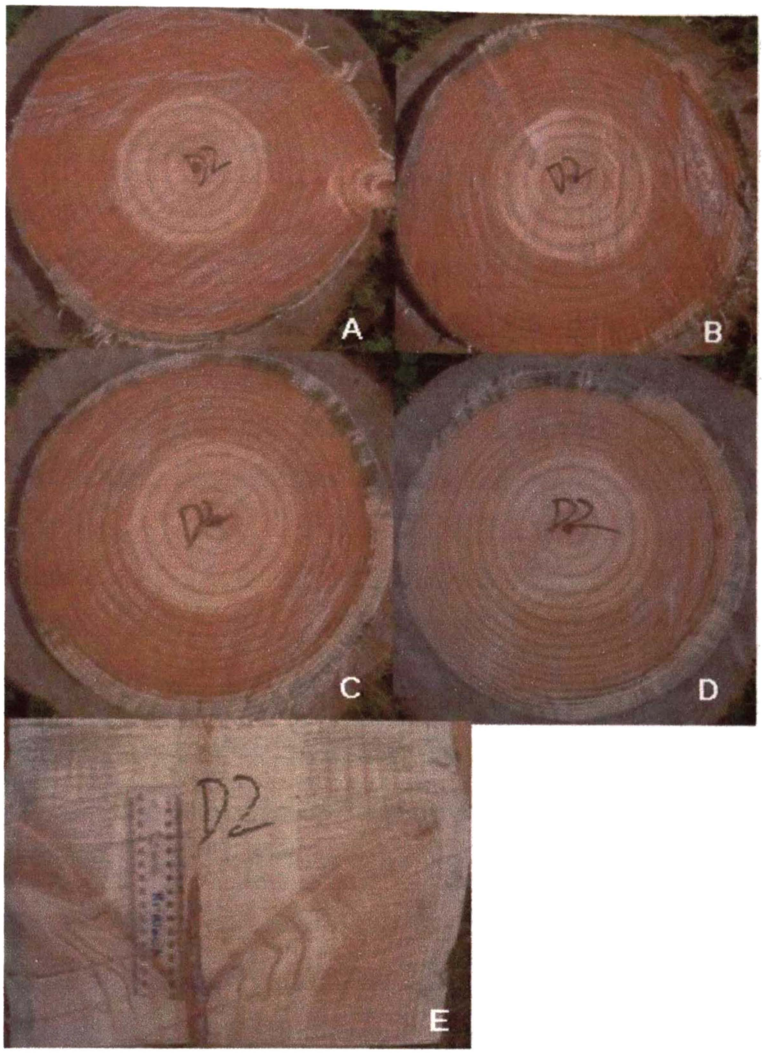


Figure 5.14: Progression of stain development in logs treated with *S. sapinea* in Colonisation Field Trial I. A= 15 days after inoculation; B = 21 days after inoculation, C= 28 days after inoculation, D= 30 days after inoculation, E = cross section showing radial stain penetration but no stain progressing from the end of the log after 30 days after inoculation.

At the end of the trial each log was sliced longitudinally as shown in Figure 5.14 (photo E). A visual measurement of sapstain was made of the longitudinal stain development from the cut end (Table 5.1). The mean daily visual longitudinal stain development for the mixed *Ophiostoma* species treatment and *S. sapinea* were 2.5mm/day and 0.25mm/day respectively.

Table 5.14: Measurements of longitudinal visual stain development at Day 30 for Colonisation Field Experiment I.

Treatment	**Mean longitudinal penetration (mm) at Day 30***
Control	No visual sapstain
Albino *O. floccosum* strains	No visual sapstain
S. sapinea	7.5 (7.5)
Mixed *Ophiostoma* species	74.8 (5.2)

* Number in parenthesis is the standard error of the mean

5.5.4.1 Fungal penetration

Visual observations of the outer surface of the logs inoculated with the mixture of *Ophiostoma* species showed vast amounts of synnemata and *Leptographium* structures from Day 9. The presence of moulds on the surface of the logs was also noted.

Cultures of all fungi were readily isolated from both stained and unstained wood pieces. Occasionally other fungi were isolated from the increment samples but were not identified further than genus level, including *Penicillium* species, *Cladosporium* species, *Trichoderma* species, *Pestalotia* species, and *Mucor* species. The inoculated sapstain fungi were in abundance on the selective media when present and the plates were not overgrown by the other fungi.

Table 5.15 shows the mean radial penetration for each sapstain fungi and each sampling point. There was a statistical difference in the radial penetration of the inoculated fungi between the days sampled (F=4.33, P<0.000). In most cases the radial penetration increased. There was also a statistical difference between the different sapstain fungi inoculated onto the wood (F=134.4, P<0.000). *O. floccosum* was the fastest sapstain fungi that penetrated the logs inoculated with the mixture of *Ophiostoma* species (*O. floccosum*, *O. ips* and *L. procerum*). At Day 3, all three fungi had penetrated into the logs to about 40mm. After 7 days, however, *O. floccosum* had penetrated the logs on average to 70mm. On subsequent sampling days, *O. floccosum* still penetrated the logs at the fastest rate. From day 9 to 15 penetration was measured only to 70mm, but at Day 18, measurements for fungal penetration were extended to 100mm. *O. floccosum* showed radial penetration to 100mm by Day 18. At the 100mm measurements, the segments were sometimes reaching the sapwood-heartwood boundary.

O. ips showed a varying amount of growth over the month period. The maximum average growth occurred on Day 21 at 77mm.

L. procerum was only able to be cultured from wood segments until Day 9. It is unsure why *L. procerum* did not colonise the logs more successfully. One suggestion is that it was out-competed by the other two fungi *O. floccosum* and *O. ips* for nutrients and space to grow. However the temperature of the trial could have influenced the growth of *L. procerum*. As previously shown *L. procerum* was unable to grow on synthetic medium or *P. radiata* specimens in the laboratory at 30°C.

High variability between the logs was evident especially for isolation of *S. sapinea*. An increase in the number of logs would lower this variability but the amount of sampling would increase and would not be practical.

Table 5.15: Mean fungal penetration (mm) and the standard error for logs in Colonisation Field Trial I.

	Mean fungal penetration (mm) and the standard error*									
	Day 3	**Day 7**	**Day 9**	**Day 11**	**Day 15**	**Day 18**	**Day 21**	**Day 24**	**Day 28**	**Day 30**
S. sapinea	0	5 ± 5b	13 ± 4.4c	5 ± 5d	13 ± 7.3e	0f	10 ± 10g	7 ± 6.7i	5 ± 5j	0l
O. floccosum	40a	70	70	70	70	97 ± 3.3	100h	100	93 ± 6.67k	97 ± 3.3k
O. ips	36 ± 3.3a	27 ± 8.8b	28 ± 15.9c	22 ± 4.4d	18 ± 3.3e	47 ± 21.9	77 ± 8.8h	57 ± 17.6	58 ± 16.9k	70 ± 5.8k
L. procerum	40a	22 ± 4.4b	17 ± 16.7c	0d	2 ± 1.7e	0f	0g	0i	0j	0l

* Values within a column with same letter so not differ significantly with a Tukey's analysis (P>0.05)

The best possible method to prevent log sapstain is to process logs as soon as possible after trees are harvested since logs are essentially unstained at harvest time (there are some exceptions to this statement, such as staining near pruning wounds in sapwood). It is not known how fast staining fungi penetrate into wood and cause significant damage when environmental conditions are most favorable. Discolouration may appear in wood only when the fungi have developed pigmentation, but fungi themselves may have penetrated far beyond that area. The results from this field trial showed that a period of 15 days of log storage during summer was enough for significant stain to develop, but the fungi were already fully penetrated into the logs.

5.5.5 Colonisation Field Experiment II – Winter penetration field trial.

A total of 48 samples were collected from the logs before spraying the treatments. One sapstain isolate (*S. sapinea)* was cultured from the outer cambium layer of one of the logs. The number of spores in the environment was low in the winter months.

Other types of fungi and yeasts were found in the samples but not identified further than genus level including *Penicillium* species, *Pestalotia* species, *Trichoderma* species, *Cladosporium* species, *Alternaria* species.

Figure 5.15 shows the average moisture content of all the logs as the month progressed as well as the rainfall for the trial period. An increase in moisture is seen over the first days and then the moisture content was maintained at about 125%. No relationship between moisture content and rainfall was evident for this trial. No visual sapstain was evident on any of the logs during the 29-day period. Whether there was no sapstain due to the temperature, moisture content or for other biological reasons needs to be explored.

The mechanism of the induction of biosynthesis of melanin in sapstain fungi and the factors that possibly contribute to melanin induction is not fully known (Kreber *et al*, 1999). Lagerberg et al (1927) reported on a study looking at the impact of moisture content on growth, examining twelve wood inhabiting fungi including *O. piliferum* and *O. piceae*. The overall trend was for very little stain development under saturated or dry conditions and for greater amounts of intense stain development at moderate moisture contents. The effect of moisture content on the development and penetration of melanin was also studied by Kreber *et al* (1999). The results of this study contradicted the study by Lagerberg *et al* (1927) as it showed that higher moisture contents produced deeper colouration than lower moisture levels. Kreber et al (1999) also showed that melanin could be controlled when pre-infected wood was kept in an oxygen free atmosphere. Eagen *et al* (1997) found that the carbon and nitrogen sources influenced fungal biomass yields and pigment colours. Conditions that mimicked the nutrients present in wood induced higher biomass and darker pigmentation. Eagen *et al* (1997) also indicated that pigmentation appeared during the active growth phase of the fungus. The data presented in this thesis extends this fact further that temperature may also be a major factor in melanin development.

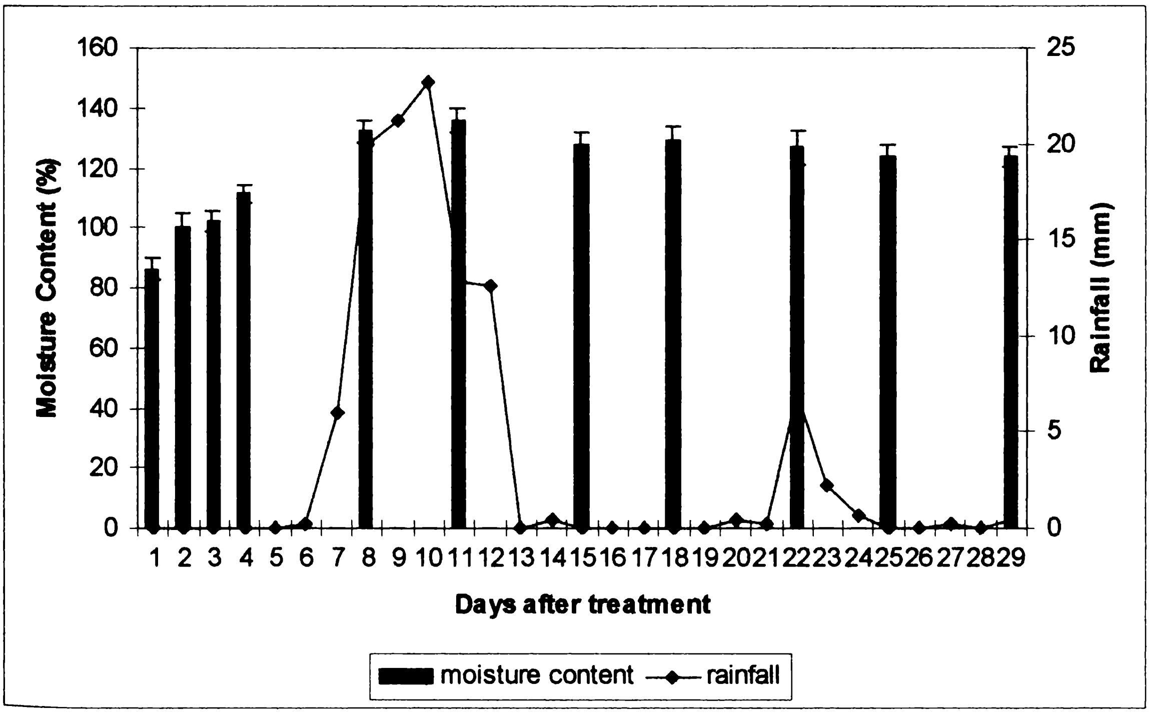


Figure 5.15: The average moisture content of logs in Colonisation Field Experiment I over a one-month period.

5.5.5.1 Fungal Penetration

Table 5.16 shows the mean radial penetration for each sapstain fungi and each sampling point. There was a statistical difference in the radial penetration of the inoculated fungi between the days sampled (F=2.31, P=0.015). In most cases the radial penetration increased. There was also a statistical difference between the different sapstain fungi inoculated onto the wood (F=99.03, P<0.000). *O. floccosum* was the fastest sapstain fungi that penetrated the logs inoculated with the mixture of *Ophiostoma* species (*O. floccosum*, *O. ips* and *L. procerum*). Isolations of *O. floccosum* were obtained from Day 1. There was a decrease in penetration of this sapstain fungus after the first four days. This may again be attributed to end infection biasing the results. Maximum penetration occurred at Day 15 and was maintained until isolation ceased on Day 29. A gradual increase in penetration is seen *L. procerum* over the first eight days. Maximum penetration for this fungus occurred on Day 8 with an average penetration of 52mm. There was a significant reduction in isolations of *L. procerum* after Day 15. The maximum penetration of 20-30mm was obtained on Day 3. There were only sporadic isolations of this fungus during the 29-day sampling period. This was in contrast to the summer trial where is obtained maximum penetration of 77mm after 21 days. These field results indicate that *O. ips* is less tolerant to colder temperatures than *L. procerum* and *O. floccosum*.

S. sapinea was not isolated from the logs until Day 2. There were only occasional isolations of this fungus during the 29 days. The maximum penetration of *S. sapinea* was on Day 11 (between 10-20mm). Comparing this to the summer trial, *S. sapinea* again did not successful colonise the logs.

Table 5.16: Mean fungal penetration (mm) and the standard error for logs in Colonisation Field Trial I.

	Mean fungal penetration (mm) and the standard error*										
	Day 1	**Day 2**	**Day 3**	**Day 4**	**Day 8**	**Day 11**	**Day 15**	**Day 18**	**Day 22**	**Day 25**	**Day 29**
S. sapinea	0a	2 ± 1.7b	2 ± 1.7c	0e	2 ± 1.7g	5 ± 2.9i	3 ± 1.7k	2 ± 1.7l	7 ± 1.7m	2 ± 1.7n	3 ± 1.7o
O. floccosum	68 ± 10.4	63 ± 11.5	53 ± 15.3d	50 ± 21.8f	67 ± 14.4h	73 ± 7.6j	80	80	80	80	78 ± 2.9
O. ips	7 ± 1.7a	7 ± 3.8b	7 ± 5.2c	0e	5 ± 1.9g	5 ± 1.8i	0k	2 ± 0.9l	0m	2 ± 0.8n	0o
L. procerum	20a	2.7 ± 4.4b	42 ± 4.4d	43 ± 4.4f	52 ± 13.6h	48 ± 20.4j	12 ± 6.0k	12 ± 11.6l	7 ± 4.4m	2 ± 1.7n	7 ± 4.4o

* Values within a column with same letter so not differ significantly with a Tukey's analysis (P>0.05)

O. floccosum was isolated from the control logs and the logs treated with *S. sapinea*. The source of infection of these logs by *O. floccosum* could have occurred through wind or insect dispersal.

As indicated in the materials and methods section (Section 5.4.5), both Colonisation Field Experiment I and II were designed to investigate the fungal stain development and fungal penetration in logs under field conditions. The University of Waikato was selected as the site for storage of the logs for ease of sampling as well as to lessen the amount of naturally occurring sapstain fungi contaminating the samples. As this is an urban site away from major forestry plantations the amount of spores within the environment were believed to be lower. Forestry sites were shown in Chapter 3 to contain greater abundance and diversity of sapstain fungi compared to other sites. This site may not reflect the situation of stain development in a forest or mill environment.

5.5.6 Microscopical analysis of growth in wood

The colonisation of *P. radiata* by sapstain fungi was analysed using microscopic techniques. No differences in hyphal penetration were observed between the different sapstain fungal species at the cellular level.

Microscopic examinations of razor blade samples of infected wood revealed that the staining was due mainly to the presence of fungal mycelia within the wood elements. Hyphae of sapstain fungi initially colonised the wood tissue via the parenchyma cells (Figure 5.16).

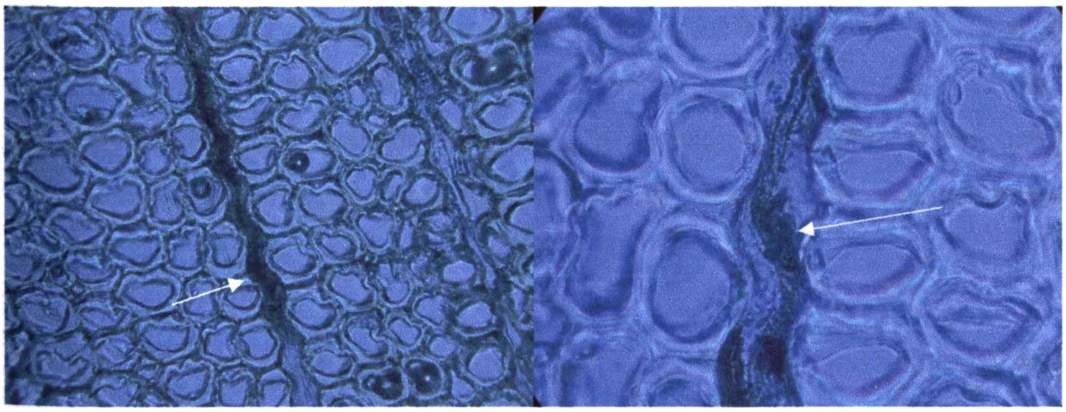


Figure 5.16: Photo of hyphae of *O. ips* growing in the parenchyma cells of *P. radiata*, white arrows indicate the presence of fungal hyphae (picture on left cross section, 200X magnification, picture on right cross section, 400X magnification).

Ballard *et al* (1984) confirmed that the fungal hyphae were initially confined to the parenchyma cells. In late stages of sapstain colonisation the fungal hyphae were fairly widespread in the sapwood tracheids (Ballard *et al*, 1982). Figure 5.17 shows the hyphae of *S. sapinea* moving from tracheid to tracheid.

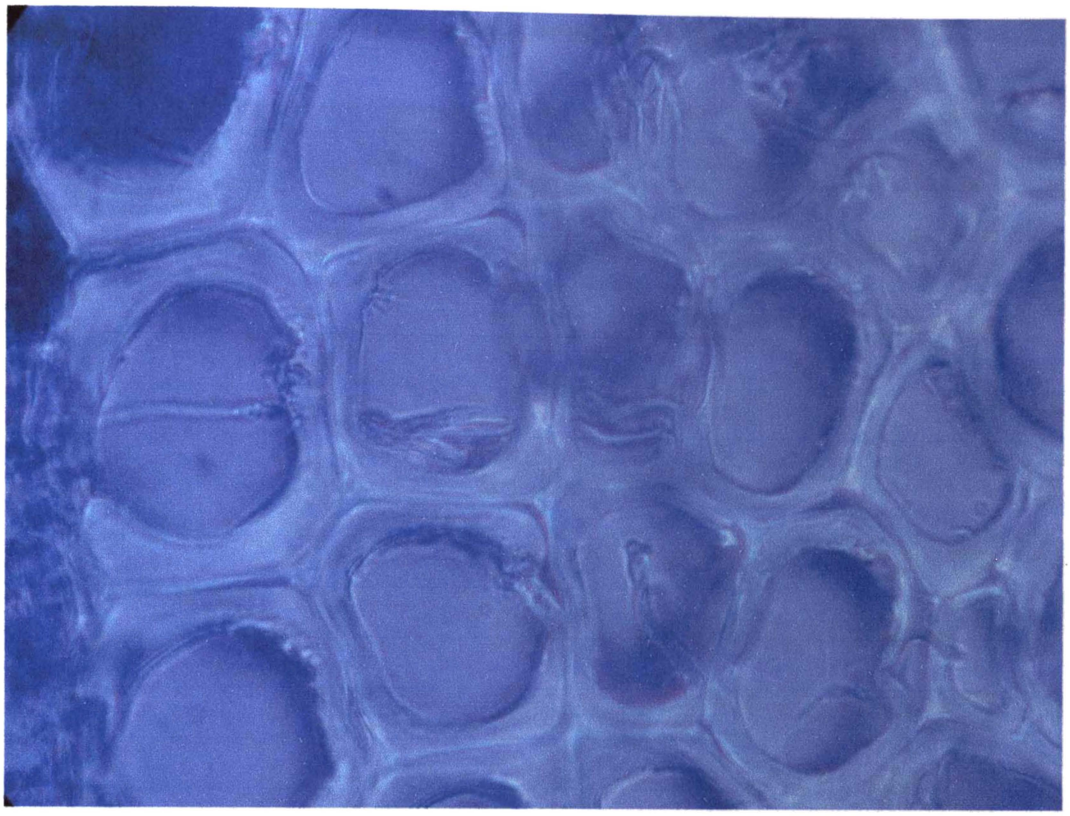


Figure 5.17: Hyphae of *S. sapinea* moving from tracheid to tracheid, arrows indicate the presence of hyphae (cross section 400 X magnification).

Liese (1970) found that the hyphae of the sapstain fungi *O. coerulum* and *O. piceae* grew between cells of *Pinus sylvestris* and *Picea abies* by two methods. The first was by penetration of border pits (between tracheids) and half border pits (connecting parenchyma cells and tracheids). The second method was by the direct penetration of the cell wall by the formation hyphal appressorium, which in turn is transformed into a tunnelling tool (transpressorium) able to penetrate through the wood wall.

Figure 5.18 and Figure 5.19 show the hyphae of the sapstain fungus *L. truncatum* growing from tracheid to tracheid and penetrating the tracheid wall by the production of an appressorium structure. The production of a fine penetration hypha about the fifth of the size of the normal hyphae allowed for the movement of the fungus between tracheids.

It was considered that the initial penetration of the tracheid wall by the appressorium was a purely mechanical action, since the hyphae were growing on the internal face of the cell walls without any enzymatic alteration of the wood structure (Liese, 1970). However, bore holes all along the wood cell wall were subsequently observed, which supposedly were due to enzymatic secretion from the appressorium that prepares the way for the transpressorium assisted by mechanical pressure (Liese, 1970).

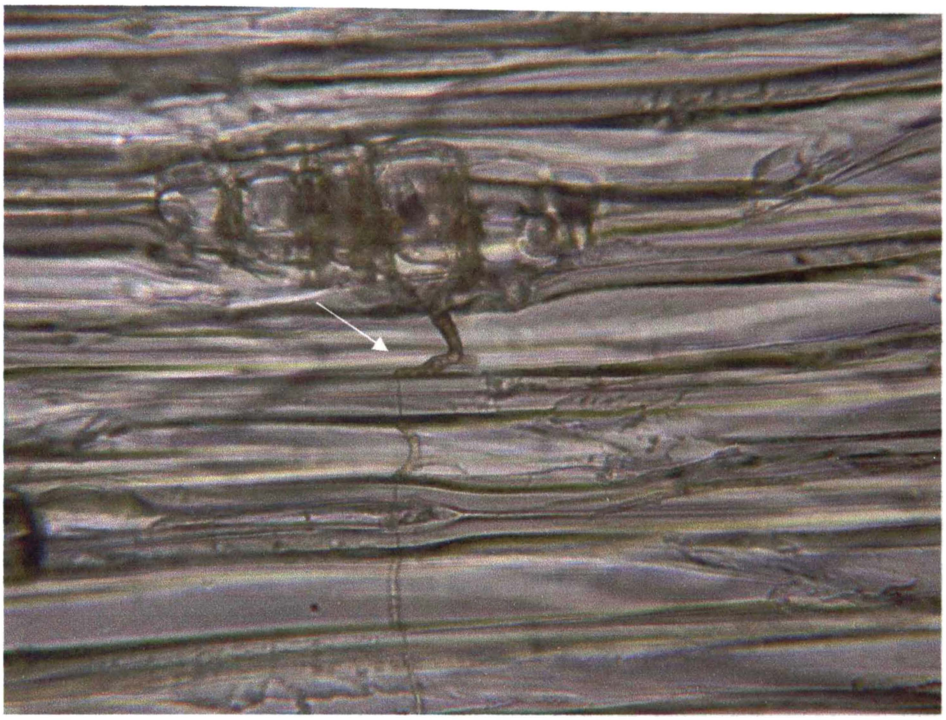


Figure 5.18: *L. truncatum* growing from tracheid to tracheid through the production of an appressorium, arrow indicates the appressorium structure (400X magnification).

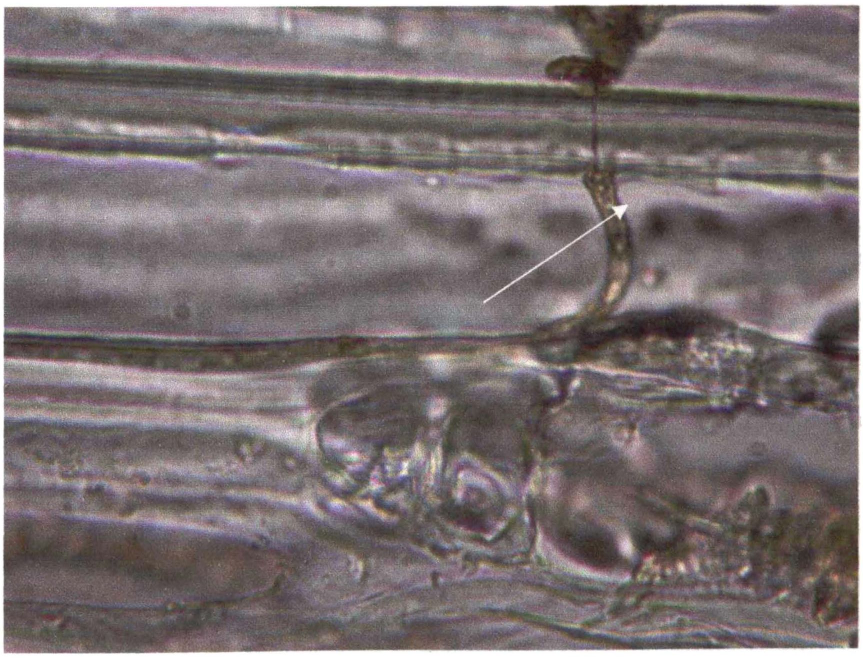


Figure 5.19: *L. truncatum* growing from tracheid to tracheid through the production of an appressorium, the arrow indicates the appressorium structure (1000X magnification).

The formation of appressorium was only evident in wood inoculated with *L. truncatum*. Wood inoculated with the sapstain fungi *S. sapinea*, *Botryosphaeria* species, *L. procerum* and *O. huntii* showed only movement of fungi from cell to cell via border pits (Figure 5.20). Blanchette *et al* (1992) found that wild type strains of the sapstain fungus *O. piliferum* did not commonly form appressorium.

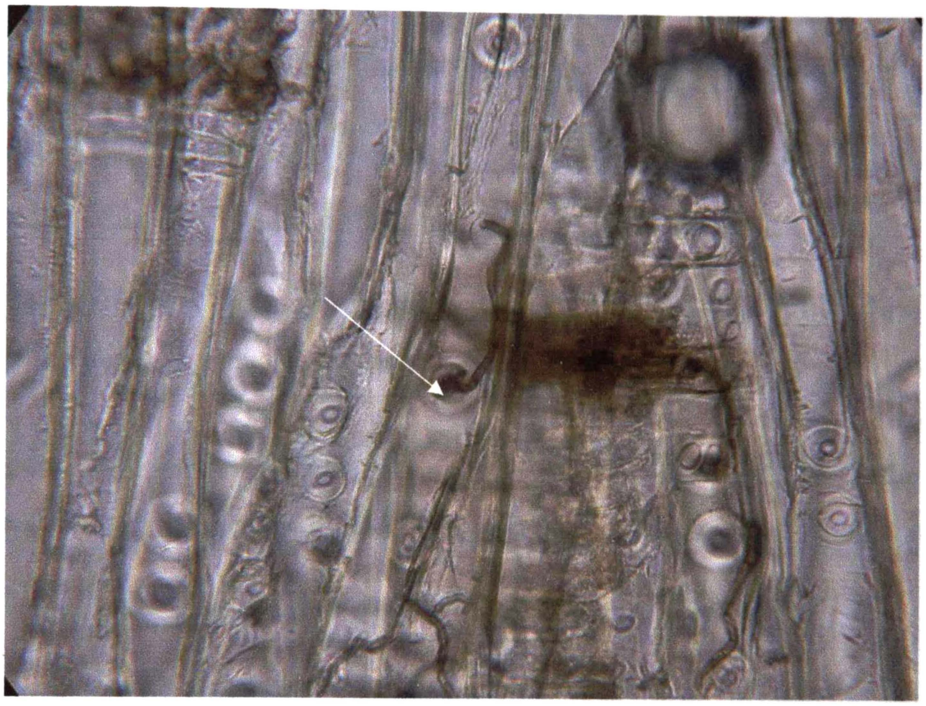


Figure 5.20: *Botryosphaeria* species moving through pit membranes, arrow indicates the hyphae moving through pit (400X magnification).

5.5.7 Summary

This research examined New Zealand isolates of four sapstain species for the impact of temperature on their growth and development in artificial media and in *P. radiata* pine specimens. This research also studied the effect of environmental factors in the field in two seasons, summer and winter, on the development sapstain and radial growth of the fungi. Table 5.17 shows a summary of the mean daily growth rates for sapstain species at three different temperatures on synthetic agar media and on specimens of *P. radiata*. The growth of all fungi on synthetic media was faster than on *P. radiata* specimens with the exception of *O. floccosum* at 15°C and 30°C. Similar trends were seen on synthetic media that were evident on wood specimens, for example, *L. procerum* was unable to successful penetrate on agar or *P. radiata* at 30°C. However some differences were also evident. *S. sapinea* was unsuccessful at penetrating *P. radiata* at 15°C but grew successfully at this temperature on agar media. *S. sapinea* and *O. ips* both had generally more rapid growth rates than *L. procerum* and *O. floccosum*. *O. ips* and *S. sapinea* are both known pathogens of conifer species which could explain their more rapid growth rates (Mathre, 1964; Chou, 1976b; Chou, 1987).

Table 5.17: Summary of mean daily growth rates for sapstain species in the laboratory on synthetic media and on *P. radiata* specimens.

	Mean growth rate (mm/day)^1					
	15°C		**23°C**		**30°C**	
Sapstain fungi	**Synthetic media**	***P. radiata***	**Synthetic media**	***P. radiata***	**Synthetic media**	***P. radiata***
L. procerum	1.9	1.2	2.8	2.5	0.5	0.2
O. floccosum	1.3	1	1.8	1.7	1	1.2
O. ips	3	1.2	4.4	2.1	6.2	2.5
S. sapinea	3.6	0.3	6.4	2.2	9.2	2

One major difference between Colonisation Field Experiments I and II was the moisture content of the logs. A reduction in the moisture content from 147% to 72% was evident over the 30-day trial period for logs in the Colonisation Field Experiment I (summer) in comparison to Colonisation Field Experiment II (winter) where an increase in moisture content was recorded from 86% to 135% for the logs over the same amount of time in the field. Moisture is thought to be a key factor influencing fungal growth in wood (Zheng *et al*, 1995). The results of Colonisation Field Experiment II showed that fungi were still able to grow and penetrate at the higher temperatures but stain did not develop. Environmental factors particularly temperature and rainfall directly affected the growth of sapstain fungi. In addition these factors influenced the moisture content all affecting sapstain development.

In conclusion, this chapter reports on the studies focussing on the response of New Zealand isolates of sapstain fungi to various temperatures and in the field environment. Temperature affected the growth of various species of sapstain fungi on synthetic media and on *P. radiata* wood. *O. ips* and *S. sapinea* grew more rapidly on both synthetic media and *P. radiata* wood specimens at higher temperatures. *O. floccosum* and *L. procerum* grew more rapidly at 23°C. In the field environment *O. floccosum* was the most successful coloniser of *P. radiata* logs in both winter and summer. Environmental factors like temperature and moisture content affected stain development. Stain developed on all treatments 15 days after inoculation in the summer field trial, however no stain developed on any treatment in the winter field trial. A number of sapstain species were also evaluated for sapstain development on laboratory specimens of *P.*

radiata. *Botryosphaeria* species produced the most extensive dark grey to black stain of all the sapstain species assessed. *O. huntii*, *O. galeiformis*, *L. truncatum* and *L. procerum* produced a light grey stain on *P. radiata*. *O. setosum*, *O. stenocerus* and *O. nigrocarpum* slightly discoloured the wood, and *O. querci* and *O. coronata* produced no appreciable stain. The colonisation of *P. radiata* by sapstain fungi was analysed under the microscope. No differences were observed with regard to the spatial distribution of the sapstain fungi. Hyphae of sapstain fungi grew in parenchyma cells and tracheids. The hyphae of the sapstain fungi passed from one wood cell to another by growing through the pit membranes or by direct cell wall penetration using an appressorium. From these results a better understanding of the biology of sapstain species is emerging. Such knowledge of the mechanisms and environmental triggers of growth and stain development will aid in the more advanced technologies to successfully control sapstain.

6 Biological control of sapstain fungi on *Pinus radiata*

6.1 Introduction

The studies of sapstain fungi in Chapter 3, Chapter 4 and Chapter 5 formed the basis of continuing investigations into the potential uses of biological control agents against these fungi.

The use of anti-sapstain chemicals is the traditional method of choice for the forest industry to control sapstain in logs and lumber. The New Zealand forest industry spends approximately NZ$5.5 million per annum on anti-sapstain chemical control to protect wood and wood products from fungal degrade during short-term storage and shipping (Deane, 1999). Toxicity concerns and the environmental effects of many chemicals used promoted research into alternative methods of control. Some markets in Europe and the United States require independent certificates to ensure that imported timber comes from sustainable managed forests and environmentally friendly processing systems. In New Zealand the forest industry must comply with the conditions of the Resource Management Act, 1991 and the Occupational Safety and Health Act, 1992. The Resource Management Act regulates the use of preservatives and there are detailed protocols for design and operation of wood treatment plants. There are concerns that residues from spillage and waste disposal are contaminating soil and waterways particularly in older designed plants. The Occupational Safety and Health Act also requires companies to establish practises to protect the health of employees involved in the use of preservatives.

Biological control using fungi, bacteria yeasts and natural products were all investigated as alternatives to chemical control. Farrell and colleagues at Sandoz Chemicals Biotech Research Corporation developed an albino biological control fungus from the sapstain fungus *O. piliferum*. This fungus, Cartapip 97®, currently marketed by AgraSol, was made by classical mating of various *O. piliferum* strains isolated in the United States. The albino isolate was blocked in the pentaketide melanin pathway by the inability to produce the intermediate scytalone (Zimmerman *et al*, 1995).

In their review of biological control, Schoeman *et al* (1999) concluded that successful biological control relies on appreciating the limitations of biological treatments and understanding the mechanisms involved. Shearer (1995) suggested that a greater knowledge about the extent of fungal competition and roles in structuring fungal communities is needed. It was suggested that the following needed to be addressed:

- Experimental field studies involving manipulation of fungal species in a variety of habitats.
- Comparative field and lab studies of the same fungal species.
- Experimental field studies involving more than two fungal species.
- Experimental and comparative studies throughout a range of environmental conditions.
- Field experiments using both antibiotic and non-antibiotic producing fungal strains or species.

The research presented in this thesis on biological control of sapstain using albino strains of *Ophiostoma* investigated Shearer's (1995) suggestions.

The chapter describes the use of albino technology to control sapstain fungi on *P. radiata*. Firstly, a review of the literature discusses the methods of control of sapstain fungi currently used. Alternative methods of control are also described with particular emphasis on development of a biological control agent using albino technology. The methods and materials, results and discussion on the utilisation of albino technology for biological control are then described.

6.2 Hypothesis, Aims and Objectives

The main hypothesis of this chapter was that newly developed albino strains of *O. floccosum*, *O. piceae* and *O. pluriannulatum* were successful at colonising and controlling wild type sapstain fungi on *P. radiata* in New Zealand and that these alternative albino *Ophiostoma* species showed an advantage over albino *Ophiostoma* species previously described.

The main aim of this chapter was to develop a greater understanding of the biology and control potential of the albino *Ophiostoma* strains developed. The first objective was to examine morphological characteristics, and colonisation of albino *Ophiostoma* species and to albino strains to wild type sapstain species in the laboratory and field environment. The second objective was to investigate selected albino *Ophiostoma* species in field trials for their anti-sapstain effect. The final objective was to examine the *in vitro* interaction and determine the possible mode of action of albino *Ophiostoma* species on wild type sapstain fungi and other wood inhabiting fungi, whether antagonistic, competitive or antibiotic.

6.3 Literature Review

6.3.1 Control of sapstain

The control of sapstain is required by the forest industry due to sapstain both lowering timber value by affecting wood quality and increasing the need for more bleaching in pulping processes. There are at least three different methods of protecting wood from sapstain currently used (Byrne, 1998) including: physical methods (kiln drying and water storage), treatment with anti-sapstain chemicals, biological control or a combination of all three methods.

6.3.1.1 Kiln Drying and water storage

In order to prevent the occurrence of sapstain in logs and timber, the wood must be quickly processed after harvesting. Kiln drying is a process that reduces the moisture content of the wood to less than 20%. The low moisture content is inhibitory to fungal growth since without water many fungal biochemical and enzymatic reactions are prevented (Zabel and Morrell, 1992). Re-wetting of the wood may promote renewed fungal growth and associated stain production.

On the other side of the moisture spectrum, to sprinkle logs or store them under water until they are required prevents the sapstain fungi colonising. The fungi are aerobic and cannot penetrate saturated wood (Findley, 1959). When these methods are not feasible, wood must be protected by other means.

6.3.1.2 Chemical control with anti-sapstain chemicals

Application of a chemical fungicide to wood and wood products is currently the method the forest industry uses to control sapstain problems in New Zealand (Wakeling, 1997). Historically, sodium pentachlorophenol (PCP) was highly efficacious in controlling sapstain (Pearson, 1988). The effectiveness of PCP as a fungicide discouraged research into other products and research on the fungi themselves. PCP's were found to be environmentally hazardous and have now been banned in most countries (Byrne, 1998). Restrictions on the use of PCP in the late 1970's meant new control methods were needed (Kang and Morrell, 2000). New chemical methods with less broad toxicity have now been developed (Byrne, 1998). None of these formulations have proven as effective for controlling stain as PCP was in the past. There are other problems associated with the use of those alternative anti-sapstain treatments such as increased costs, higher requirements for worker sensitivity, cold weather handling complications and high aquatic toxicity (Seifert, 1993). There is also a possibility that the temporary status of these compounds as acceptable pesticides could be subject to review.

Antisapstain chemicals inhibit fungal growth by the formation of a thin barrier that prevents the germination of fungal spores on the wood substrate (Zabel and Morrell, 1992). These chemicals are applied to the surface of lumber by dipping into tanks or by spraying. Control of sapstain is more difficult for logs than sawn lumber as stated by Kreber *et al* (2001) in the following ways:

- In logging the wood surface can get punctured with ensuing fungal infection of the punctures
- An inherently uneven log surface is prone to damage before and after anti-sapstain treatment due to the handling of logs with heavy machinery
- Existing fungal pre-infections occur as a consequence of delays between harvesting and application of anti-sapstain treatment.

There are about 15 chemical formulations registered for sapstain control in New Zealand (FRI, 1997). The formulations most commonly used by the industry are based on the quaternary ammonium compound didecyldimethylammonium chloride (DDAC) and Copper-8-quinolinolate in the formulation Cutrol 375 (Fernz Timber Protection). DDAC is used with the co-biocide 3-iodo-2propynyl butyl carbamate (IPBC) in the formulation NP1 (Kop-Coat Inc.).

6.3.1.3 Biological control

Biological control is now an important part of research into the protection of wood products, due to the increasing environmental and consumer related unacceptance of chemical protection. Biological control agents can be grouped under the headings: antibiosis, competition, induced resistance, and myco-parasitism. These modes of action are not mutually exclusive; typically the control agent can work in more than one way (Kay, 1997).

6.3.1.3.1 Modes of antagonism for biological control agents

Biological control agents may simply operate by occupying the ecological niche of the target organism so preventing its establishment. This method is referred to as primary resource capture followed by competition. Competition is the active demand for nutrients, oxygen, water or for space by two or more individuals of the same or different species for the same resource (Dix and Webster, 1995). Primary colonising fungi are important biological agents in competitive interactions as they can occupy the niche and utilise the nutrients before subsequent colonisation of other organisms. Each fungus may defend (via antibiosis or other means) its already colonized resource or one fungus may challenge another and engage in secondary resource capture or the two fungi may merely intermingle with one another, with no discernible deleterious effects to either organism (Klepzig and Wilkens, 1997).

Many microorganisms produce toxic substances in response to contact with another microorganism that allows a microbial colonist to compete for the specific resource (Schoeman *et al*, 1999). Traditionally, microbial antibiotics were split into non-volatile metabolites, which diffuse through a liquid phase and volatile metabolites, which spread as vapours (Schoeman *et al*, 1999).

The third biological control method, myco-parasitism and the production of extracellular enzymes, implies that the target organism has already colonised the substrate. These mechanisms are probably less important for biological control of sapstain fungi, which are primary colonisers of wood (Morrell, 1999).

6.3.1.3.2 Screening for Biological control agents

Laboratory assays are essential to screen large numbers of potential biological control agents. Dual culture agar assays are the most commonly used screen for potential biological control agents (Bruce and Highley, 1991; Croan and Highley, 1991) and show the interactions between the biological control agent and the target fungus that include competition, antibiosis and parasitism. Laboratory assays, while fast, simple and cost effective, do not necessarily reflect condition on wood surfaces and may provide misleading results (Benko and Highley, 1990; Kreber and Morrell, 1993). A better understanding of the nature of microbial interactions on wood surfaces is needed (Smouse *et al*, 1999). The successful laboratory biological control studies may eventually aid in understanding the mechanisms of biological control, however only field demonstration can prove biological control.

6.3.1.3.3 Successful Biological Control agents

There are hundreds of papers demonstrating biological control in laboratory assays. Problems of transferability to the field were encountered for many biological control agents owing to poor colonisation either caused by poor nutrient status of wood or the presence of other resident flora (Bruce *et al*, 1990).

Successful biological control was demonstrated by the yeast *Debaryomyces* species on *Pinus sylvestris* (Payne *et al*, 2000; Payne and Bruce, 2001), *Gliocladium roseum* on hemlock and fir (McAfee and Gignac, 1997; Dawson-Andoh and Lovell, 2000), *Phialemonium curvatum* on *Populus tremuloides* (Hiratsuka and Chakravarty, 1999), Basidiomycetes on a variety of wood species (Blanchette *et al*, 1996) and *Gliocladium viride, Trichoderma hamatum, T. harzianum, Trichothecium roseum*, on *P. radiata* (Kay, 1997; Vanneste *et al*, 2002) and by albino *O. piliferum* strains on *Pinus resinosa* (Behrendt *et al*, 1995a,b). The use of albino technology is discussed in more detail in this Chapter.

Research was carried out by several groups on the use of natural products to control sapstain fungi. Though these strategies will not be discussed in this thesis, the reader is directed to the demonstration of the potential use of essential oils in the following references. Dawson-Andoh *et al* (2000) studied the inhibitory effect of essential oils on sapstain in lab based assays and Hill *et al* (1997) and Vanneste *et al* (2002) demonstrated the potential of essential oils, particularly pine oil based treatments, in controlling sapstain fungi in New Zealand on *P. radiata* logs and sawn timber at mill sites.

Many of the field trials with biological control agents produced inconsistent results that may imply an inability to control sapstain fungi or difficulty of colonisation on certain wood substances or under specific environmental conditions (Smouse *et al*, 1999). Smouse *et al* (1999) suggested that more research is need with mixtures of biological control agents. This chapter will focus on biological control of solid wood products with single strains as well as mixtures of albino *Ophiostoma* species.

6.3.2 Albino *Ophiostoma* species as biological control agents

The finding of the melanin production pathway for *O. piliferum* was achieved from research on an albino or colourless *O. piliferum*. Albino *O. piliferum* strains were originally developed for pitch reduction in the pulp and paper industry. In the late 1980's, Bear Island Paper Company in the United States, noticed seasonal pitch disturbances in their southern yellow pine thermochemical pulp operation. Sandoz Chemicals Biotech Research Corporation (Charlotte, North Carolina) and their colleagues at the University of Minnesota identified *O. piliferum* as the organism responsible for the decrease in extractive content and this reduces pitch problems (Brush *et al*, 1994). Pitch is a major problem for the pulp and paper industry due to deposit forming on machines and the lowered quality of the finished material (Blanchette *et al*, 1992).

By classical genetic mating using ascospores of *O. piliferum* and then subsequent selection procedures, the albino or colourless strains were developed (Blanchette *et al*, 1992). This work resulted in the development of the current commercial product, the fungal inocula Cartapip® 97. A suspension of fungal spores of Cartapip 97 was applied to the wood immediately after chipping (typically 1 x 10^{11} colony forming units per metric ton wet weight of wood) and within 10-14 days after inoculation, as much as 50% of the pitch was removed (Blanchette *et al*, 1992; Farrell *et al*, 1993).

The albino strain Cartapip 97 was blocked to synthesise melanin at the point before scylatone synthesis in the pentaketide pathway (Zimmerman *et al*, 1995). Melanin and perithecial development were restored when mycelia of the albino strain was grown on media supplemented with an extract of spent culture fluid derived from a pigmented strain of *O. piliferum*. The extract contained scylatone and other intermediates of the pentaketide pathway. Pure scylatone also restored hyphal pigmentation and perithecial development (Zimmerman *et al*, 1995). The decreased amount or lack of melanin in the albino strains obtained in these studies did not appear to inhibit the aggressiveness or growth characteristics of the albino fungi. Microscopy studies of inoculated loblolly pine showed that the albino fungi grew similarly to the wild type fungi colonising the parenchyma cells and resin ducts after 21 days incubation (Blanchette *et al*, 1992).

Further investigations using Cartapip 97, showed this organism to be successful in controlling sapstain, principally on *Pinus resinosa* and southern yellow pine in North America (Blanchette *et al*, 1992; Farrell *et al*, 1993; Behrendt *et al*, 1995a,b). These studies demonstrated that applying Cartapip 97 to freshly cut logs allowed the inoculated strain to preferentially colonise the sapwood, thereby, capturing nutrient resources and inhibiting subsequent colonisation by organisms that would occupy the same ecological niche as the dark staining fungi.

In Germany, Schmidt and Müller, (1996) showed similar success with the same strain of the colourless *O. piliferum* on *Pinus sylvestris* sawn timber, and debarked logs controlling sapstain and rot species.

White-McDougall *et al* (1998) developed albino strains from the sapstain species *O. piceae* using the methodology developed in the creation of Cartapip 97. From isolates of the New Zealand sapstain fungal survey discussed in Chapter 3, albino isolates of *O. floccosum*, *O. piceae* and *O. pluriannulatum* were developed. Albino isolates *O. pluriannulatum* were demonstrated to be effective by this thesis research and in collaboration with colleagues at the University of Minnesota in field trials in New Zealand on mature *P. radiata* logs to control sapstain (Held *et al*, 2003).

6.4 Materials and Methods

6.4.1 Albino Development

Strains of three *Ophiostoma* species, *O. floccosum*, *O. piceae* and *O. pluriannulatum*, isolated from *P. radiata* in the survey of sapstain fungi in New Zealand (Chapter 3) were used in mating studies to develop albino strains. Albino strain development was completed at the University of Minnesota. Albino strains were constructed for each species by single ascospore isolations and subsequent mating of strains according to the methods described in Zimmerman *et al* (1995).

Table 6.1 shows the parentage of each albino *Ophiostoma* strain that was used in this thesis research, with accession numbers of those isolates deposited in the Australian Government Analytical Laboratories (AGAL). Tester strains of known mating types were used to determine mating capabilities of unknown strains as described in Chapter 2 (Section 2.4.2.3). Mating of strains of *O. pluriannulatum* were carried out by transferring A and B strains to opposite sides of a Petri dish containing 1.5% malt extract agar and allowing them to grow together. Perithecial formation occurred in the center of the plate where the two cultures merged. The mating of strains of *O. piceae* and *O. floccosum* was carried out by inoculating malt extract agar amended with sterile *P. radiata* twigs and/or wood chips. Media amended with wood was inoculated with one mating type two days later. Ascospores were harvested up to four weeks after mating by dispersing spore droplets into sterile water. A dilute ascospore suspension was streaked onto agar media. Spores were germinated and individually transferred to fresh agar media. Thousands of single spore isolates were evaluated for mycelium that lacked pigmentation. Selected isolates were incubated at 5°C for up to four weeks. Isolates that remained colourless after this "cold treatment" were further tested in challenge experiments. Selected isolates were also used in additional mating studies to obtain a large number of colourless strains.

6.4.2 Evaluation of colony characteristics and intrinsic growth rate assays

The colony morphology of each albino strain was examined and compared to wild type strains of the same species. Cultures were examined after being incubated for two weeks at room temperature on malt extract agar. The development of darkened mycelia was also assessed for each albino after growth at 30°C.

Growth studies of selected isolates were conducted on malt extract agar and malt yeast extract agar. Isolates were first grown on malt extract agar for approximately one week. Discs 6mm in diameter were cut from the actively growing colony margins and placed at the centre of 90 mm plastic Petri dishes with three replicates per isolate, and incubated in darkness at the following temperatures 6°C, 15°C, 23°C and 30°C. Growth measurements at 6°C were suspended after the first cultures were assayed as the growth rates were too slow to get an accurate measurement. Whether a culture was capable of growth at 6°C was then noted.

The first measurements of the colony diameter were taken after two days incubation at right angles. The second and subsequent measurements were taken after one to four days depending on the speed of growth of the particular isolate. Growth measurements ceased after 30 days or when the colony edge was within 5 mm of the edge of the dish. The average growth rate was calculated as the mean radial increment per day, using the two measurements per plate, the daily measurements and the three replicate plates per isolate. Statistical analysis was performed as analysis of variance and Tukey's test for comparisons of means using Minitab 12 for Microsoft Windows.

Table 6.1: Parentage of each albino *Ophiostoma* strains used in this thesis.

Albino Isolate	Parentage	AGAL Accession number (strains deposited on the 16/08/00
O. floccosum		
F13	68 x 123	NM00/12246
F40	68 x 123	NM00/12247
F80	F25 x 123	NM00/12491
F93	F3 x 36	NM00/12492
F124	F106 x 26	Not deposited
F132	F106 x 26	Not deposited
F145	F106 x 36	Not deposited
O. pluriannulatum		
3410	6NZ-175 x 6NZ-173	NM00/12252
5040	6NZ-175 x 6NZ-173	NM00/12251
7073	5040 x 6NZ-150	NM00/12493
O. piceae		
OPC 422	OPC 1 x 272	NM00/12248
OPC 703	OPC 542 x 272	NM00/12489
OPC 846	OPC 400 x 272	Not deposited
OPC 848	OPC 400 x 272	Not deposited
OPC 849	OPC 400 x 272	Not deposited
OPC 1	501 x 125	Not deposited

6.4.3 Laboratory colonisation studies of albino *Ophiostoma* strains

The materials and methods related laboratory colonisation experiments were presented in Chapter 5 (Section 5.4.3). The albino strains F13 and F40 were selected for testing and the results are present in this chapter.

6.4.4 Albino Colonisation Field Trial I (Summer 2000/2001)

The materials and methods related to this trial were presented in Chapter 5 (Section 5.4.4). Three logs were inoculated with a mixture of two albino *O. floccosum* strain F13 and F40 were included in this trial and the results are presented in this chapter. The combined fungal species were inoculated at approximately 1 x 10^5 colony forming units per ml with a total of four litres of inoculum sprayed evenly onto the three logs.

6.4.5 Albino Colonisation Field Trial II (Winter 2001)

The materials and methods related to this trial were presented in Chapter 5 (Section 5.4.5). A total of three logs were inoculated with an albino *O. pluriannulatum* strain 3410 and the results are presented in this chapter. The fungal species were inoculated at approximately 5 X 10^5 colony forming units per ml with a total of four litres of inoculum sprayed onto the three logs.

6.4.6 Biological Control Field Trial I (November 1997)

Biological Control Field Trial I was established in mid November 1997 and continued until April 1998 at the Kinleith Mill in the Central North Island of New Zealand. The site for the trial was an elevated storage site within 400 metres of Kinleith Forest, highly exposed to wind and sun. Six-metre logs were cut from trees approximately 24 years old and were obtained from Kinleith Forest and transported to the site the day after harvesting. Logs were then cut into four pieces of 1.5 metres each and randomly laid out into piles for treatment.

Albino isolates chosen for this field trial were strains that were most effective at controlling sapstain fungi in laboratory challenge experiments undertaken at the University of Minnesota. Sixteen different albino *O. pluriannulatum* strains (3410, 3640, 4630, 4650, 4680, 4700, 4790, 4890, 5030, 5040, 5080, 5980, 6010, 6110, 7014, 7036) were inoculated onto logs and a set of logs sprayed with only water was established as a control. The albino strains were prepared by inoculating 1 two-litre flask containing one litre of malt yeast extract media with each culture and grown in a shaking incubator at 25°C for two to three days. The excess medium was removed by centrifugation and the fungal suspensions were resuspended in one litre of 100mM Tris-HCl buffer at pH 7. Approximately 1 X 10^8 colony forming units per mL for each albino strain were then resuspended in four litres of tap water. Albino strains and a control (tap water only) were sprayed onto nine logs per treatment using a hand garden sprayer. After six months in the field (November to May, the New Zealand summer and autumn months), the logs were assessed for coverage of sapstain on four internal faces of each log. All logs were sliced into five pieces (30 cm intervals) and each face was assessed immediately after being sprayed with water. The percentage sapstain coverage on each face was estimated by two groups of two assessors each. The assessors estimated the total amount of coverage of blue, grey and black stain at 5% intervals on the entire face of the wood discs taken from the internal sapwood of the logs, scoring the amount of stain from 0-100%. Statistical analysis was performed as analysis of variance and Tukey's test for comparisons of means using Minitab Version 12 for Microsoft Windows.

6.4.7 Biological Control Field Trial II (Winter 1998)

Biological Control Field Trial II was established in June 1998. The site and log parameters were the same as for Biological Control Field Trial I. Ten logs per treatment were inoculated with the most successful albino strains from Biological Field Trial I. The treatments included the albino *O. pluriannulatum* strains 5040, 4650 and 3410 and control treatment water. The albino strains were prepared by inoculating 10 two litre flasks containing one litre of malt yeast extract media with cultures and grown in a shaking incubator at 25°C for two to three days. The excess medium was removed as in Biological Control Field Trial I. Approximately 1 X 10^9 colony forming units per ml albino strain were then resuspended in 50 litres of water. A commercial spray unit was used for log inoculation as compared to hand garden sprayers in Biological Control Field Trial I. A three log by three log replicate block design was used for this trial. After three months the logs were assessed for stain development as in the first trial.

6.4.8 Biological Control Field Trial III (Summer 2000)

This log trial was established in February 2000 (New Zealand Summer) at Kinleith Forest, Tokoroa, in the Central North Island of New Zealand. The site was situated just off a forestry road within the Kinleith Forest that was previously used as a small storage site. There was no predominant wind affecting the site.

The logs were harvested from approximately 24-year-old *P. radiata* trees sourced from the same site within the Kinleith Forest. Logs of six metres with an average diameter of 380mm were cut, debarked and transported to the site the day after harvesting. They were then cut into four pieces of 1.5metres each and randomly laid out on *Eucalyptus* species log bearers.

For this trial, treatments consisted of mixtures of different albino *Ophiostoma* species inoculated onto the 1.5m long lengths with 9 replicates per treatment. Treatments C, E, H, K, M, S, T and X contained one strain of an albino *O. pluriannulatum*, *O. floccosum* and *O. piceae* in equal concentrations. The albino strains were prepared by inoculating two 2-litre flasks with each albino culture and grown in a shaking incubator at 25°C for three to four days as described in for Biological Control Field Trial I (Section 6.4.7). The excess growth medium was removed by centrifugation and the fungal suspensions were resuspended in 100mm Tris-HCl at pH 7 to make up to one litre. A total spore concentration of colony forming units per litre in a total of 4 litres of water was applied for all albino treatments. Treatment Y consisted of the albino *O. pluriannulatum* strain 3410 made up from a freeze-dried product (Chapter 2, Section 2.8) to a spore concentration of 1 X 10^7 colony forming units per mL. A control treatment (A) consisting of water only was also including in this trial. All treatments were sprayed onto the logs with a hand garden sprayer. All surfaces of the logs were sprayed until there was a liquid run off.

The treatments were coded as follows:

A = Control (water only)
C = Albino *O. piceae* strain 422, Albino *O. floccosum* strain F93, Albino *O. pluriannulatum* strain 7073
E = Albino *O. piceae* strain 422, Albino *O. floccosum* strain F80, Albino *O. pluriannulatum* strain 7073
H = Albino *O. piceae* strain 422, Albino *O. floccosum* strain F80, Albino *O. pluriannulatum* strain 3410
K = Albino *O. piceae* strain 422, Albino *O. floccosum* strain F93, Albino *O. pluriannulatum* strain 3410
M =Albino *O. piceae* strain 703, Albino *O. floccosum* strain F80, Albino *O. pluriannulatum* strain 7073
S = Albino *O. piceae* strain 703, Albino *O. floccosum* strain F93, Albino *O. pluriannulatum* strain 7073
T = Albino *O. piceae* strain 703, Albino *O. floccosum* strain F80, Albino *O. pluriannulatum* strain 3410
X =Albino *O. piceae* strain 703, Albino *O. floccosum* strain F93, Albino *O. pluriannulatum* strain 3410
Y = Freeze dried *O. pluriannulatum* strain 3410, reconstituted with water

Debris samples were taken at the establishment of the trial from small branches, cones, needles and bark from around the site. The samples were surface sterilized and plated on to selective media (Media 4 and Media 6) for sapstain fungi as described in Chapter 2 (Section 2.3).

After five months in the field the logs were assessed for coverage of visual sapstain on three internal surfaces of each log. All logs were sliced into four equal lengths (approximately 40cm intervals) with a chain saw. Four independent assessors estimated the amount of visual sapstain coverage on the three inner faces of each log. The total amount of blue, grey and black stain at 5% intervals on only the sapwood of the transverse surface of the face was estimated, scoring the amount of stain from 0-100%. The most dominant stain colour according to the stain intensity scale (Table 6.2) was also estimated for each log face. Measurements were taken of the diameter and the percentage of sapwood for each log.

To determine how much of the sapwood was colonized by the fungal treatment or wild type sapstain fungi, disc samples, from internal log faces were taken from a random selection of logs in each treatment. Small segments of wood approximately 5x5x3cm were removed with a chisel from each disc. The samples were processed in the laboratory as described in Chapter 2 (Section 2.3).

Table 6.2: Stain intensity value scale.

Stain intensity Value	Description
0	No stain
1	Palest grey stain
2	Pale grey stain
3	Grey stain
4	Dark grey stain
5	Black stain

Mean percentages and standard error of the estimated amounts of visual sapstain and the intensity of stain were calculated for each treatment. Statistical analysis was performed as analysis of variance and Tukey's test for comparisons of means using Minitab 12 for Microsoft Windows.

6.4.9 Biological control field trial IV (Summer 2000/2001)

This second trial was established in December 2000 at the beginning of the New Zealand Summer in Kinleith Forest but at a different location than the Biological Control Field Trial III. This site was sheltered and deeper into Kinleith Forest and surrounded by a mature 24-year-old *P. radiata* plantation. *P. radiata* logs were harvested, cut and inoculated as in the previous trial. The logs had an average diameter of 410mm and were again cut into 1.5 metre lengths.

Treatments consisted of two controls, one treated with water and the other not. Three single strain albino treatments were included in this trial, albino *O. pluriannulatum* strains 5040 and 3410 and an albino *O. piceae* strain OPC 703. The final treatment was a mixture of two albino *O. floccosum* strains F40 and F13. All albino treatments consisted of approximately 1 x 10^6 colony forming units per mL. All fungal treatments were in a freeze-dried powder (Chapter 2, Section 2.8). The albino treatments were added to four litres of water, well mixed and sprayed with a garden hand sprayer on the nine logs per treatment. All surfaces of the logs were sprayed until run off. The logs were set up into six treatment piles due to the limitation of space at the site.

Samples were taken at the establishment of the trial from the outer surfaces of the logs prior to inoculation. The samples were surface sterilized and plated on to selective media for sapstain fungi as described in Chapter 2 (Section 2.3).

Logs were assessed after five months as in Biological Control Field Trial III with three assessors estimating the total coverage visual sapstain on the sapwood of three transverse faces per log. The total amount of blue, grey and black stain at 5% intervals on only the sapwood of the transverse surface of the face was estimated, scoring the amount of stain from 0-100%. The most dominant stain colour according to the stain intensity scale (Table 6.2) was also estimated for each log face.

A more extensive fungal colonisation analysis was performed for this trial. Samples were taken to estimate the amount of sapstain and albino fungi colonising each log. Five samples of wood were taken randomly from each of the 3 faces per log for all of the treatments for analysis. These five samples were brought back to the laboratory, and sampled as described in Chapter 2 (Section 2.3).

Percentages for colonisation of wood samples by the albino fungi or sapstain fungi were determined by dividing the number of successful isolations of the fungi by the number of isolation attempts per logs (average of 15 samples per log were taken). If there were more than one identical fungus isolated from a sample it was still counted only as a positive isolation with no consideration given to the multiple isolation.

Mean percentages and standard error of the estimated amounts of visual sapstain, the intensity of stain and fungal colonisation were calculated for each treatment. Each treatment log in this field trial, though sampled from the same log pile, was analysed as a replicate using analysis of variance and Tukey's test ($P = 0.05$) for comparisons of means using Minitab 12 for Microsoft Windows.

6.4.10 *In vitro* interactions of Albino *Ophiostoma* strains and other wood inhabiting fungi including sapstain species

Observations of pigment production and the interaction between albino *Ophiostoma* strains and other wood inhabiting fungi including sapstain species were carried out using simple competition plate bioassays. The interactions of albino *Ophiostoma* strains against sapstain fungi (*S. sapinea* isolate D35, *L. procerum* isolate 1852, *O. querci* isolate 1688 and *O. ips* isolate P36) and wood inhabiting fungi (*E. nigrum*, *C. cladosporioides*, and *A. alternata*) in dual culture were studied on malt extract agar in 90mm Petri plates. The plates were inoculated with albino *Ophiostoma* strains by placing 6mm diameter plugs (taken from the periphery of actively growing cultures) at the margin. They were allowed to grow for two days at room temperature. After two days, 6mm diameter mycelial plugs of the antagonistic fungus (either a sapstain species or a wood inhabiting fungus) were inoculated on the plate opposite to the albino *Ophiostoma* strain and incubated at room temperature for up to 14 days. Three replicates of each fungal interaction were done. The interaction between the two fungi was noted, according to one of the following criteria:

- The albino *Ophiostoma* strain completely overgrew the other fungal colony.
- The other fungus overgrew the albino *Ophiostoma* strain.
- The two colonies met with no overgrowth or inhibition zone formation.
- An inhibition zone formed between the two colonies.

The observations of the interaction between the fungal colonies were used in a supportive role to indicate the mode of antagonism.

6.5 Results and Discussion

The first part of this results and discussion section describes two field trials that were established by the candidate prior to starting the doctoral research and in association with colleagues at the University of Minnesota. The results are included as they formed the essential background work for selection of the albino strains used in this doctoral thesis research. The results of both field trials are also presented in Held *et al* (in press), a copy of this publication is provided in Appendix 1.

6.5.1 Biological Control Field Trial I

After six months, logs inoculated in the field with 17 different albino *Ophiostoma* strains were assessed. Figure 6.1 shows the mean coverage of sapstain for each treatment. With analysis of variance of the data and outliers removed from the mean stain values, log treatments with eight albino *O. pluriannulatum* strains (5040, 3410, 4890, 4650, 5080, 4680, 6110, and 6010) showed significantly less stain than the control (sprayed with only tap water) logs. There was a group of albinos that were not significantly different to the control logs and a group (7036, 7014, and 4630) that were statistically more stained than the controls.

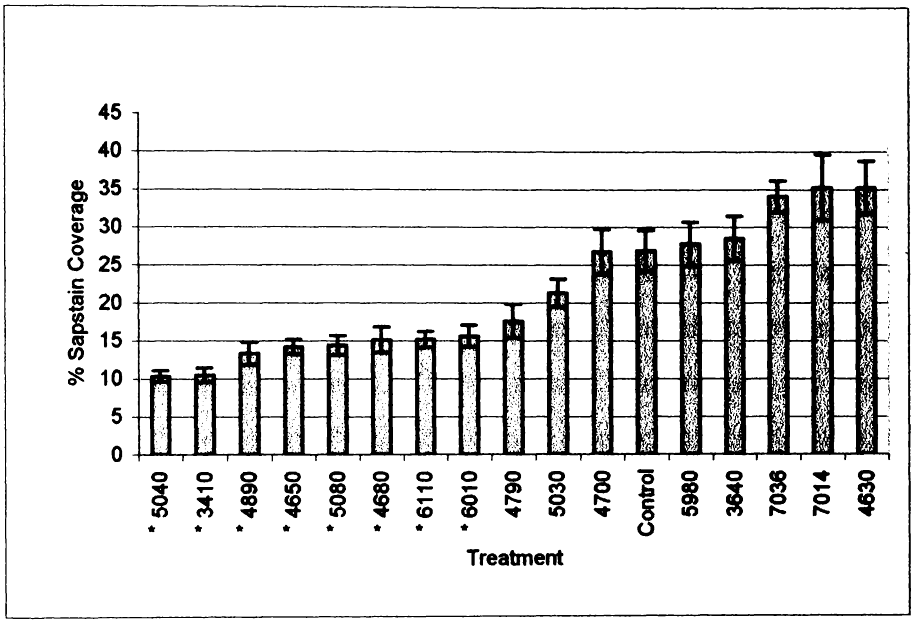


Figure 6.1: Visual evaluation of sapstain on logs from Biological Control Field Trial I with outlying data points removed. The * indicates treatments that were statistically different to the control where p=0.05.

6.5.2 Biological Control Field Trial II

In a second log trial established in June 1998, albino *O. pluriannulatum* strains 3410, 5040 and 4650 from the first trial were again inoculated onto *P. radiata* logs. Figure 6.2 shows the mean percent sapstain coverage according to treatment. This trial showed that albino *O. pluriannulatum* strain 3410 had significant reduction in the amount of sapstain in comparison to control logs. The other two albino *O. pluriannulatum* strains (5040 and 4650) were not statistically different from the control logs in the amount of sapstain.

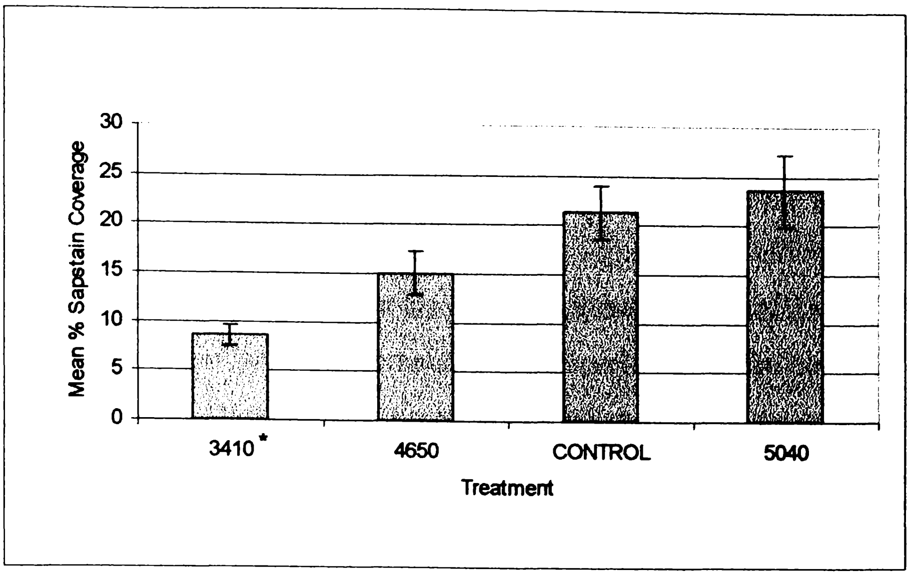


Figure 6.2: Visual evaluation of sapstain in logs from Biological Control Field Trial II showing the mean percentage of sapstain coverage for treatments. The * indicates treatments that were statistically different to the control where p=0.05.

Results from Biological Control Field Trial I and II showed significant differences among strains used for biological control of sapstain. The effectiveness of controlling sapstain varied among strains tested. These results demonstrate the need for more investigation into the biology of the albino strains in order to select for more effective control fungi. The second log trial was done to evaluate several isolates from the first log trial at a different time of year (June to November). Although isolates 4650 and 5040 did not perform significantly better than the control in this particular study, isolate 3410 showed significant sapstain control from the control logs.

Additional field experiments were warranted to evaluate more albino strains. In Biological Field Trial II, strains 4650 and 5040 may not have performed as well as in Biological Field Trial I due to prior contamination of logs with wild type sapstaining fungi during harvest and transport. Another possibility is that these strains were not ideally suited for the environmental conditions occurring at the time of the study. Growth studies in Chapter 5, showed that sapstain fungi grew as a function of temperature and moisture content. Additional field trials were needed to elucidate the crucial environmental factors that could affect the success of the albino strains and result in more effective control.

6.5.3 Evaluation of colony characteristics and *in vitro* growth rates of albino *Ophiostoma* strains

Albino isolates of *O. floccosum*, *O. pluriannulatum* and *O. piceae* varied in morphological traits (Table 6.3). The albino strains of *O. floccosum* F13 and F40 both formed synnemata on both agar and wood. The synnemata had darkly pigmented stalks characteristic of the wild type *O. floccosum* strains (Figure 6.3). Albino strains of F13 and F40 were of dark appearance when grown on malt extract agar at 23°C. This was in part due to the large number of synnemata, which developed in the colonies, a typical characteristic of the wild type *O. floccosum* strains when cultured on malt extract agar. At ambient temperatures, the hyphae of albino *O. floccosum* strains were white with no melanisation but when these fungi were grown at 30°C brown pigmented hyphae were evident for all strains. All albino strains of *O. piceae* and *O. pluriannulatum* were white with no synnemata formation and induced no melanisation at 30°C.

Table 6.3: Morphological characteristics of albino *Ophiostoma* strains used in this thesis.

Albino isolate	Synnemata	Pigmentation of hyphae	Induction of pigmentation at 30°C	Pigmentation in media
O. floccosum				
F13	Yes	White	Yes	Yellow
F40	Yes	White	Yes	Yellow
F80	No	White	Yes	Yellow
F93	No	White	Yes	Yellow
F124	No	White	Yes	Yellow
F132	No	White	Yes	Yellow
F145	No	White	Yes	Yellow
O. pluriannulatum				
3410	No	White	No	No
5040	No	White	No	No
7073	No	White	No	No
O. piceae				
OPC 422	No	White	No	No
OPC 703	No	White	No	No
OPC 846	No	White	No	No
OPC 848	No	White	No	No
OPC 849	No	White	No	No

Figure 6.3: Common growth characteristics of albino *O. floccosum* strains. A=Albino *O. floccosum* strain F40 growing from inoculated *P. radiata* slithers, showing characteristic brown synnemata and white mycelia. B= Albino *O. floccosum* strain F13, showing characteristic brown synnemata (20X magnification). C= Albino *O. floccosum* strain F124, showing characteristic white mycelia (10X magnification)

Growth responses of albino and wild type *O. floccosum*, *O. pluriannulatum* and *O. piceae* under varying temperature treatments were quantified by measuring the radial colony extension on malt extract agar and malt yeast extract agar. The growth rates of each species were determined at 15°C, 23°C and 30°C. The growth patterns of the albino were similar to that of the wild type strains tested, indicating that the albino mutation had no affect on the growth temperature response of the fungus. However the albino *O. pluriannulatum* strains and *O. piceae* strains were significantly faster growing at some temperatures compared to the wild type strains (Table 6.4).

Table 6.4 shows the growth rates for wild type and albino strain of *O. floccosum* on different media at the three temperatures. Growth rates with the same letter following the standard error figure were not significantly different according to Tukey's pairwise comparisons. The growth rate range for the albino *O. floccosum* strains was between 1.6 to 2.0mm/day compared with 1.7 to 2.0mm/day for wild type of *O. floccosum* strain at 23°C. The slowest at all temperatures was albino *O. floccosum* strain F80. Like the wild type *O. floccosum* strains, the growth rates of the albino *O. floccosum* strains were fastest at 23°C compared to 15°C and 30°C.

Table 6.4: Linear growth rate of albino *O. floccosum* strains at three different temperatures.

Albino *O. floccosum* isolate and media type	**Radial growth rate (mm/day)^1** **15°C**	 **23°C**	 **30°C**
F13 M	1.4 ± 0.01ijkl	1.9 ± 0.04opqr	0.7 ± 0.02bc
F13 YM	1.2 ± 0.00fghi	1.8 ± 0.10opqr	0.9 ± 0.08cde
F40 M	1.4 ± 0.02hijk	1.9 ± 0.02qr	0.9 ± 0.01cd
F40 YM	1.3 ± 0.02ghij	1.9 ± 0.00opqr	1.2 ± 0.03fghi
F80 M	0.9 ± 0.04cd	1.6 ± 0.06klmn	0.7 ± 0.07bcd
F80 YM	0.7 ± 0.05bcd	1.6 ± 0.02klmn	0.8 ± 0.14cd
F93 M	1.4 ± 0.00hijk	1.9 ± 0.06opqr	0.7 ± 0.09bcd
F93 YM	1.4 ± 0.01hijk	1.9 ± 0.01opqr	1.0 ± 0.03def
F124 M	1.5 ± 0.01jklm	1.9 ± 0.01pqr	0.6 ± 0.01bcd
F124 YM	1.4 ± 0.03hijk	1.9 ± 0.04opqr	0.8 ± 0.02cd
F132 M	1.4 ± 0.00hijk	1.9 ± 0.00pqr	0.5 ± 0.01ab
F132 YM	1.3 ± 0.03ghi	1.9 ± 0.01pqr	No data
F145 M	1.5 ± 0.02jklm	2.0 ± 0.07r	0.5 ± 0.09a
F145 YM	1.4 ± 0.02hijk	1.9 ± 0.03opqr	0.8 ± 0.02bcd
Wild type *O. floccosum* isolates and media type			
68 M	1.4 ± 0.01ijk	2.0 ± 0.03qr	0.9 ± 0.00cde
68 YM	1.2 ± 0.01fghi	1.7 ± 0.02mnop	1.1 ± 0.01efg
148 M	1.2 ± 0.03fgh	1.8 ± 0.02nopq	0.9 ± 0.02cd
148 YM	1.2 ± 0.02fghi	1.7 ± 0.03lmno	0.7 ± 0.00abc
J2004 M	1.4 ± 0.00ghijk	1.8 ± 0.01nopq	1.3 ± 0.04ghij
J2004 YM	1.2 ± 0.03fgh	1.8 ± 0.03nopq	0.8 ± 0.02cd

^{1}Mean radial growth rate (mm) per 24 hours ± standard error. Values within the table with same letter so not differ significantly with a Tukey's analysis (P>0.05).

Table 6.5 shows the growth rates for wild type and albino strain of *O. piceae* on different media at the three temperatures. Growth rates with the same letter following the standard error figure were not significantly different according to Tukey's pairwise comparisons. There was a statistical variation in the growth rates of different Albino *O. piceae* strains (F=14.37, P<0.000). However wild type *O. piceae* strains did not significantly differ in growth rates (F= 7.68, P=0.82). There was no significant difference between the two media for the albino *O. piceae* and the wild type *O. piceae* strains (F=3.02, P=0.85; F=0.82, P=0.370 respectively). There was a significant difference for both albino and wild type strains of *O. piceae* at the three different temperatures (F= 1870.1, P<0.000 and F=741.7, P<0.000 respectively).

Albino *O. piceae* strains were faster growing at 23°C with a range from 2.6 to 3.0 mm/day compared to than the wild type strain, which ranged from 2.1 to 2.6mm/day. One wild type *O. piceae* strain 144 was unable to grow at 30°C in contrast the other wild type and albino strains of *O. piceae* grew at 30°C at about 20% of their rate of growth at 23°C.

Table 6.5: **Linear growth rate of albino *O. piceae* strains at three different temperatures.**

	Radial growth rate (mm/day)[1]		
Albino *O. piceae* isolate and media type	**15°C**	**23°C**	**30°C**
OPC 422 M	1.9 ± 0.01bc	2.8 ± 0.00hij	0.5 ± 0.04lmn
OPC 422 YM	1.9 ± 0.01abc	2.7 ± 0.01hi	0.4 ± 0.00lmn
OPC 703 M	2.0 ± 0.01bcde	2.9 ± 0.03ij	0.3 ± 0.01lm
OPC 703 YM	1.9 ± 0.01abc	2.6 ± 0.02gh	0.7 ± 0.05n
OPC 846 M	2.0 ± 0.02bcd	3.0 ± 0.03j	0.5 ± 0.04mn
OPC 846 YM	2.0 ± 0.02bc	3.0 ± 0.02j	0.5 ± 0.00mn
OPC 848 M	2.2 ± 0.03def	2.9 ± 0.03ij	0.2 ± 0.01kl
OPC 848 YM	1.9 ± 0.02bc	2.7 ± 0.03hij	0.7 ± 0.01n
OPC 849 M	2.3 ± 0.03def	2.9 ± 0.02ij	No data
OPC 849 YM	2.0 ± 0.01bc	2.8 ± 0.01hij	0.3 ± 0.00klm
Wild type *O. piceae* isolate and media type			
J1566 M	2.0 ± 0.13bc	2.6 ± 0.04gh	0.5 ± 0.02mn
J1566 YM	1.9 ± 0.04bc	2.3 ± 0.02ef	0.5 ± 0.02mn
144 M	2.0 ± 0.02b	2.5 ± 0.02fg	0k
144 YM	2.0 ± 0.02bc	2.5 ± 0.02fg	0k
174 M	1.8 ± 0.02ab	2.1 ± 0.19cde	0.3 ± 0.02lm
174 YM	1.7 ± 0.08a	2.3 ± 0.03ef	0.3± 0.00lm

[1] Mean radial growth rate (mm) per 24 hours ± standard error. Values within the table with same letter so not differ significantly with a Tukey's analysis (P>0.05).

Table 6.6 shows the growth rates for wild type and albino strains of *O. pluriannulatum* on different media at the three temperatures. Growth rates with the same letter following the standard error figure are not significantly different according Tukey's pairwise comparisons. Albino *O. pluriannulatum* strains and wild type *O. pluriannulatum* strains had faster growth rates at 30°C then 15°C or 23°C. Albino and wild type *O. pluriannulatum* strains were faster growing on malt extract agar than yeast malt agar at all temperatures. Albino *O. pluriannulatum* strains had faster linear growth rates than the wild type strains at all temperatures. The range of daily growth at 15°C for the albino strains was 1.6 to 2.0mm/day compared with 1.2 to 1.8 for the wild type. At 23°C the range of growth for the albino strains was 2.2 to 2.8mm/day compared with 1.7 to 2.4 for the wild type. The same trend was evident at 30°C where the ranges in growth were from 2.6 to 2.8 mm/day and 1.8 to 2.8mm/day for albino and wild type *O. pluriannulatum* strains, respectively.

Table 6.6: Linear growth rate of albino *O. pluriannulatum* strains at three different temperatures.

	Radial growth rate (mm/day)[1]		
Albino *O. pluriannulatum* isolate and media type	***15°C***	***23°C***	***30°C***
3410 M	2.0 ± 0.01efg	2.8 ± 0.01m	2.8 ± 0.01m
3410 YM	1.7 ± 0.01cde	2.3 ± 0.01hij	2.6 ± 0.02kl
5040 M	1.8 ± 0.01ef	2.7 ± 0.04l	2.8 ± 0.01m
5040 YM	1.7 ± 0.01cde	2.4 ± 0.00jk	2.6 ± 0.10kl
7073 M	1.8 ± 0.03de	2.6 ± 0.01k	2.8 ± 0.05m
7073 YM	1.6 ± 0.01bcd	2.2 ± 0.01hi	2.6 ± 0.03kl
Wild type *O. pluriannulatum* isolate and media type			
151 M	1.4 ± 0.05a	1.8 ± 0.06de	2.3 ± 0.06hij
151 YM	1.6 ± 0.04abc	2.1 ± 0.04gh	1.8 ± 0.06de
847 M	1.8 ± 0.02de	2.4 ± 0.02kj	2.3 ± 0.05ij
847 YM	1.7 ± 0.01de	2.4 ± 0.02kj	2.7 ± 0.06lm
962 M	1.5 ± 0.01ab	2.1 ± 0.03h	2.8 ± 0.02m
962 YM	1.2 ± 0.03	1.7 ± 0.03bcde	2.4 ± 0.03jk

[1] Mean radial growth rate (mm) per 24 hours ± standard error. Values within the table with same letter so not differ significantly with a Tukey's analysis (P>0.05).

The albino *Ophiostoma* species grew at a slightly greater rate on both malt extract media and malt yeast extract media than the wild type strain. Frederick *et al* (1999) studied unmelanised mutants of *Gaeumannomyces* species and wild type fungi and found that the unmelanised mutants were also able to grow faster than the melanised fungi. Henson *et al* (1999) proposed that the loss of melanisation in fungi is compensated by increased enzyme production, favouring fungal colonisation and invasion. Research with unmelanised mutants of *Gaeumannomyces* species showed that enzyme production was inversely correlated with hyphal pigmentation (Henson *et al*, 1999). The unmelanised mutants had more extracellular enzyme activity than the wild type.

6.5.4 Laboratory colonisation studies of albino *Ophiostoma* strains

The albino *Ophiostoma* strains grew similarly to wild type isolates, with rapid growth on non-sterile wood specimens. The development of stain on wood specimens of the internal surface of *P. radiata* after 30 days incubation was measured. No stain was visible on the albino *O. floccosum* treated specimens sampled after three and seven day's incubation. After 30 days incubation still no stain was visible on the albino *O.*

floccosum stains F13 and F40 at 15°C and 23°C but an average of 3mm of stain developed at 30°C.

The radial penetration of the mixture of albino *O. floccosum* strains (F13 and F40) on *P. radiata* wood specimens at three temperatures is shown in Figure 6.4. The albino *O. floccosum* strains grew to 10mm after three days at all temperatures. After seven days the fastest average growth of *O. floccosum* was 35mm at 23°C. After 30 days the maximum average growth of *O. floccosum* on the laboratory specimens was 70mm at 15°C. These results indicated a greater lag period at 15°C and 30°C than at 23°C for this fungus. Statistically (P=0.05) the growth of the albino *O. floccosum* strains was faster at 15°C than 23°C or 30°C after 30 days incubation.

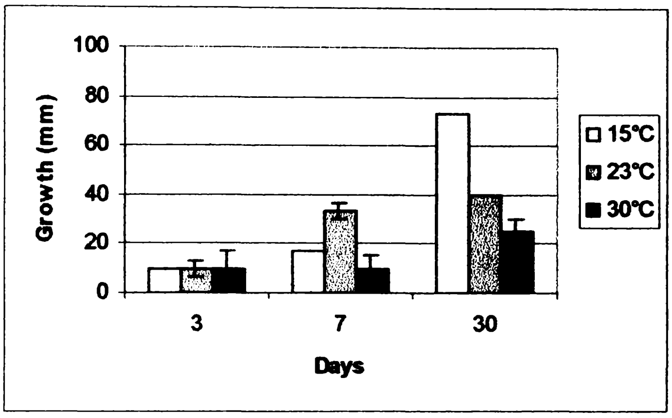


Figure 6.4: Radial growth of a mixture of albino *O. floccosum* strains F13 + F40 on *P. radiata* specimens over a period of 30 days at various temperatures.

The radial penetration of the mixture of albino *O. pluriannulatum* strains (3410) on *P. radiata* wood specimens at three temperatures is shown in Figure 6.5. No stain developed on any *P. radiata* specimen inoculated with albino *O. pluriannulatum* (strain 3410) at any time point or at any temperature. Albino *O. pluriannulatum* (strain 3410) colonised the wood specimens between 5 and 10mm after three days at all temperatures. After 30 days the maximum average growth of was albino *O. pluriannulatum* 55mm at 30°C, 25mm at 23°C and 38mm at 15°C. There were no statistical differences between any of the means at any temperature or any time point for albino *O. pluriannulatum*.

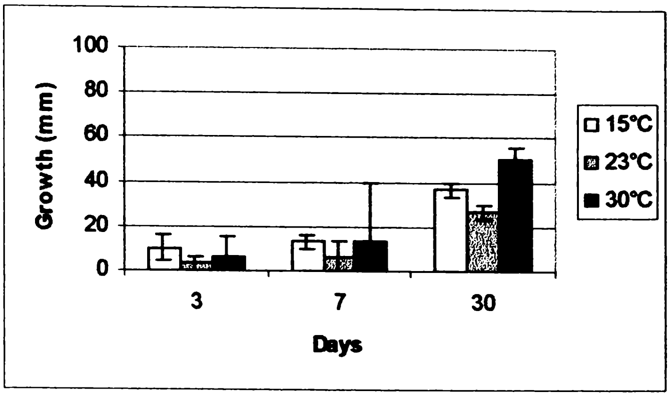


Figure 6.5: Radial growth of albino *O. pluriannulatum* strain 3410 on *P. radiata* specimens over a period of 30 days at various temperatures.

6.5.5 Albino Colonisation Field Trial I (Summer 2000/2001)

The penetration results for wild type *Ophiostoma* species were presented in Chapter 5. The albino strains used in the two field trials are included in this chapter.

Visual sapstain developed on the logs inoculated with albino *O. floccosum* strains and this stain increased over time. Figure 6.7 shows the increase in sapstain development on the log number A1, from Day 15 to Day 30. Sapstain appeared on the cross-section of the disc around the majority of the circumference extending radially towards the heartwood (Figure 6.7). At the end of the trial each log was sliced longitudinally as shown in Figure 6.7 (photo E). No longitudinal visual sapstain appeared on any log inoculated with the albino *O. floccosum* strains.

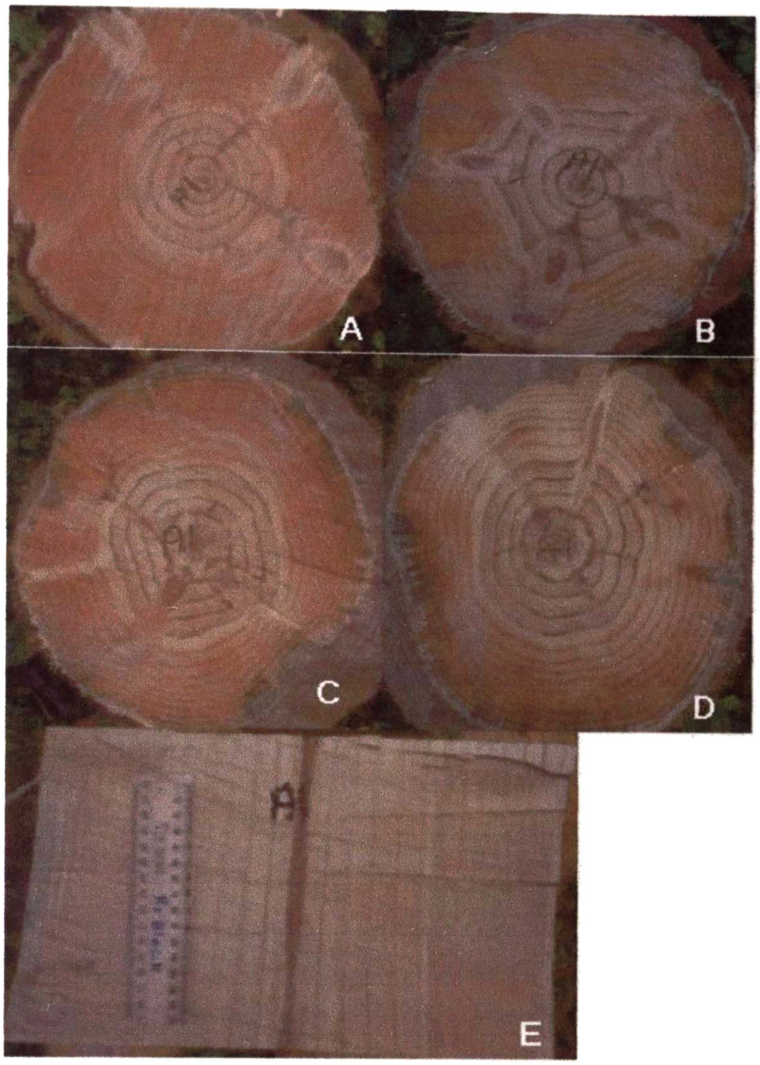


Figure 6.6: Progression of stain development in logs treated with albino *O. floccosum* strains F13 and F40 in Colonisation Field Trial I. A= 15 days after inoculation; B = 21 days after inoculation, C= 28 days after inoculation, D= 30 days after inoculation, E = cross section showing radial stain penetration but no stain progressing from the end of the log after 30 days after inoculation.

Table 6.7 shows the fungal penetration of a mixture of albino *O. floccosum* stains (F13 and F40) and the wild type *O. floccosum*. There was no difference in growth between the albino and wild type strain for this summer trial. Both treatments penetrated radially to a maximum of 100mm after 18 days.

Table 6.7: Radial penetration of a mixture of albino *O. floccosum* stains (F13 and F40) and the wild type *O. floccosum*.

	Mean fungal penetration (mm) and the standard error									
	Day 3	Day 7	Day 9	Day 11	Day 15	Day 18	Day 21	Day 24	Day 28	Day 30
Albino *O. floccosum* (F13 and F40)	40	70	70	70	70	100	100	97 ± 3.3	100	93 ± 6.7
Wild type *O. floccosum*	40	70	70	70	70	97 ± 3.3	100	100	93 ± 6.7	97 ± 3.3

6.5.6 Albino Colonisation Field Trial II (Winter 2001)

No visual sapstain developed on the albino *O. pluriannulatum* treated logs in this winter trial. The results of the mean fungal penetration of albino *O. pluriannulatum* (strain 3410) 29 days after inoculation in the field are shown in Figure 6.7. After one day the albino strain had penetrated into the logs. The higher penetration value at Day 1 (18mm) compared to Day 2 (10mm) could be associated with longitudinal infection, even though this biscuit sample was taken 10cm into the log. Rapid fungal penetration occurred between Day 4 and Day 8. This fungus had penetrated to the sapwood/heartwood boundary (80mm) by Day 15.

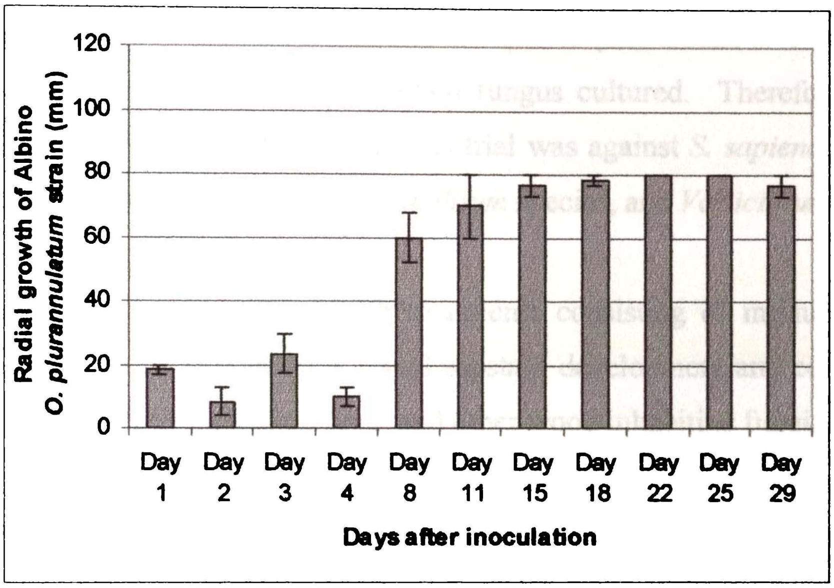


Figure 6.7: Average penetration of Albino *O. pluriannulatum* strain 3410 after 29 days incubation on logs.

The colonisation rates of the albino *O. floccosum* strains F40 and F13 were comparable to wild type *O. floccosum* strains in both laboratory and field trials. Blanchette *et al* (1992) showed that Cartapip 58, an albino strain of *O. piliferum*, colonised the wood in a similar manner to the wild type *O. piliferum*. The greatest concentrations of hyphae were in the ray parenchyma cells and the resin canals. A wild type *O. pluriannulatum* strain was not added to the laboratory or field experiments so a comparison cannot be made. Growth on agar media however showed that the albino *O. pluriannulatum* strains were faster growing than the wild type strains. Wild type *O. pluriannulatum* is not widely found in New Zealand and only ten isolates were found from the survey of sapstain fungi in New Zealand presented in Chapter 3. The ability of albino *O. pluriannulatum* strains to colonise *P. radiata* successfully suggests that it is not an inability of the fungus to grow on *P. radiata* that limits its abundance in New Zealand.

The production of stain evident on the logs inoculated with albino *O. floccosum* strains F13 and F40 in the field in Albino Colonisation Field Trial I will be discussed later in this chapter.

6.5.7 Biological Control Field Trial III (Summer 2000)

Debris samples including branches, twigs, needles and cones were taken from the site of Biological Control Field Trial I at the establishment of the trial. A total of 10 samples were taken from this site and two isolates of *S. sapinea* (one isolate from dead needles and one from a cone) were the only sapstain fungus cultured. Therefore, the initial target for biological control of the logs in this trial was against *S. sapiena*. Other fungi isolated included *Trichoderma* species, *Penicillium* species, and *Verticillium* species.

After five months, logs inoculated with treatments consisting of mixtures of albino *Ophiostoma* strains were assessed for visual sapstain development and colonisation by albino *Ophiostoma* species, sapstain fungi and other wood inhabiting fungi.

Independent assessors estimated the percent of visual sapstain coverage for three faces of each log in this trial. The mean percentage coverage for each treatment is shown in Figure 6.8. A large variation in sapstain development was evident for this trial. The percent sapstain coverage ranged from a log from Treatment H with 7% stain to a log from Treatment C with 80% stain. There was significant log-to-log variation within treatments. Treatment C had a variation from 7% to 63% in visual stain coverage. There was no significant difference in the amount of visual sapstain coverage between the different treatments (F=0.97, P= 0.468).

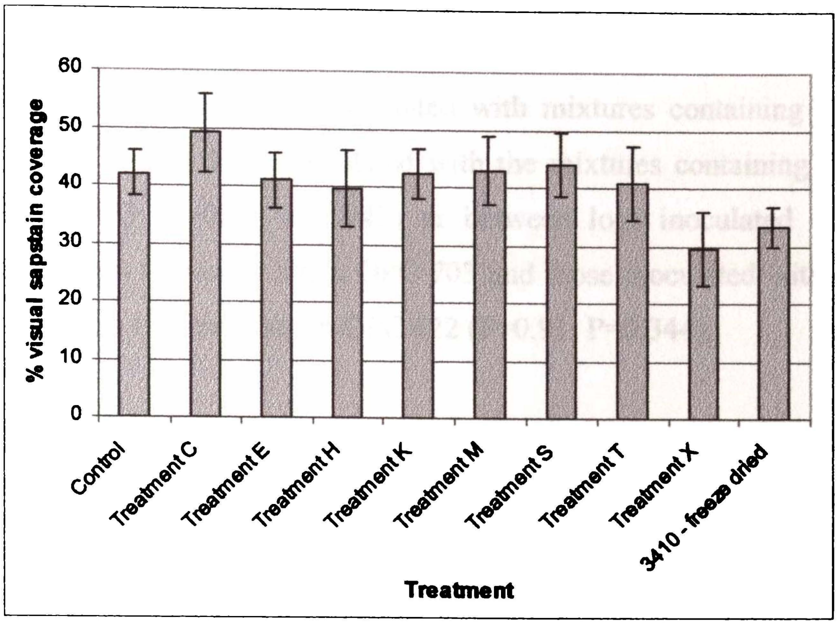


Figure 6.8: The mean percentage visual sapstain coverage as assessed for logs after five months in the field inoculated with mixtures of albino *Ophiostoma* strains.

The stain intensity was scored for Biological Control Field Trial I and the mean stain intensity values for each treatment are shown in Figure 6.9. The mean intensity ranged from 3.6 (grey) to 4.4 (dark grey) for treatment K and treatment M, respectively. There was no statistical difference in the intensity of the stain between treatments (F=1.127, P= 0.353).

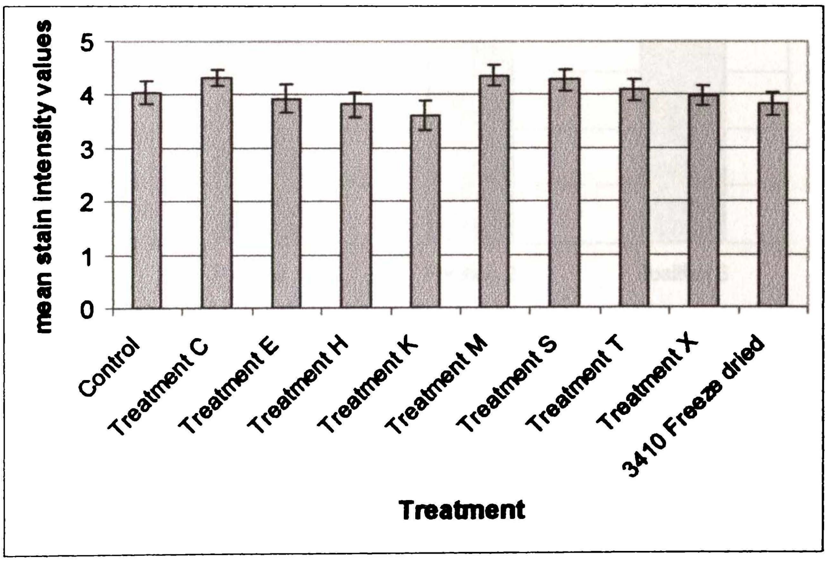


Figure 6.9: The mean stain intensity values as assessed for logs after five months in the field inoculated with mixtures of albino *Ophiostoma* species.

The data was then analysed and grouped according to individual strains in the mixtures. There was no statistical difference between the amounts of visual stain on logs for any one strain. Analysis of variance showed no difference between logs inoculated with the albino *O. pluriannulatum* strain 3410 compared to those inoculated with mixtures containing the albino *O. pluriannulatum* strain 7073 (F=2.23, P=0.140). There was no statistical difference between logs inoculated with mixtures containing the albino *O. floccosum* strain F80 and those inoculated with the mixtures containing the albino *O. floccosum* strain F93 (F=0.01, P=0.942) or between logs inoculated with mixtures containing the albino *O. piceae* strain OPC 703 and those inoculated with the mixtures containing the albino *O. piceae* strain OPC 422 (F=0.91, P=0.344).

The visual stain coverage data was compared according to the position within the log (Figure 6.10). The logs were sliced into four pieces and each cut face was assessed. The three cut faces were at the following measurements: 37.5cm (Position 1), 75cm (Position 2) and 112.5cm (Position 3). There was a statistical difference between the amount of visual stain in the middle of the log compared to each end of the log (F=4.46, P= 0.012), indicating within log variation for coverage of visual stain. Position 2 (middle of the log) was statistically more stained than Position 1 or Position 3 (approximately 40cm into the log on either end)

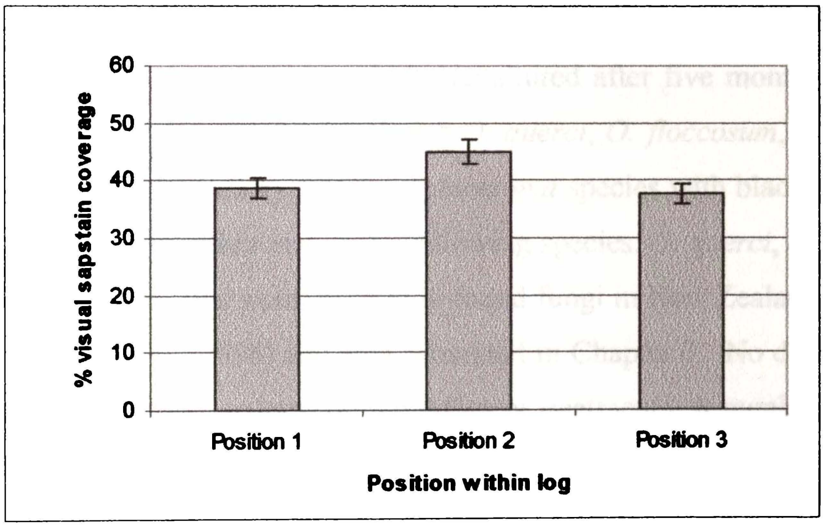


Figure 6.10: Percent visual sapstain coverage according to position within each log for Biological Control Field Trial I.

The total diameter and heartwood diameter of each log was measured. The amount of visual stain coverage was correlated to the total diameter of each log. Figure 6.11 shows that there was no relationship between the diameter of the log and the percent visual stain coverage for Biological control Field Trial 1 (R^2=0.1032). The percentage sapwood in each log face was calculated from the total diameter and heartwood diameter measurements and compared with the percent visual stain coverage. There was no correlation between these factors (R^2= 0.007).

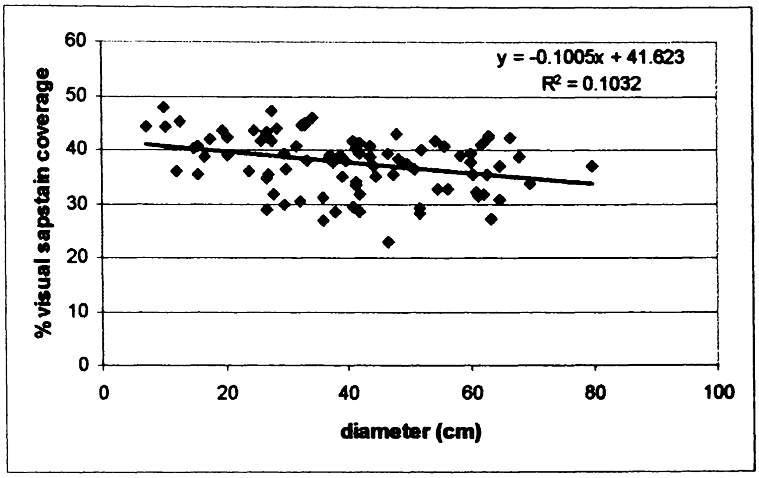


Figure 6.11: The diameter of each log compared with the percent visual stain coverage for Biological Control Field Trial I.

Table 6.8 shows the fungi colonising the Biological Control Field Trial III from culturing after five months in the field. In the majority of treatments, all three albino species inoculated within a mixture were recultured at the end of the trial with the exception of Treatment C, Treatment E and Treatment T. For Treatment C only the albino *O. floccosum* in the mixture could be recultured after five months in the field. The sapstain fungi found in this trial included *O. querci*, *O. floccosum*, *S. sapinea* and *Ophiostoma* species with black synnema. *Ophiostoma* species with black synnema was unidentified further but is either one of the following species: *O. querci*, *O. piceae* or *O. setosum*. All of these species were commonly found fungi in New Zealand according to the survey of Farrell *et al* (1998) and data presented in Chapter 3. No differences were evident in the sapstain fungi colonising the different treatments. A number of albino *O. pluriannulatum* and albino *O. piceae* strains were isolated from the control treatment in this experiment.

Table 6.8: Albino *Ophiostoma* species, sapstain species and other fungi isolated and identified from Biological Control Field Trial III.

Treatment	Albino Ophiostoma species	Sapstain fungi	Other fungi
Treatment A - Control	Albino *O. pluriannulatum*, Albino *O. piceae*	*Ophiostoma* sp. with black synnema, *S. sapinea*	*Trichoderma* sp., *Verticillium* sp., *Penicillium* sp.
Treatment C - OPC 422 F93 7073	Albino *O. floccosum*	*Ophiostoma* sp. with black synnema	*Trichoderma* sp., *Rhizopus* sp., *Verticillium* sp., *Penicillium* sp.
Treatment E - OPC 422 F80 7073	Albino *O. pluriannulatum* Albino *O. floccosum*	*Ophiostoma* sp. with black synnema, *O. querci*, *S. sapinea*, *O. floccosum*	*Rhizopus* sp., *Trichoderma* sp., *Verticillium* sp., *Penicillium* sp.
Treatment H - OPC 422 F80 3410	Albino *O. floccosum*, Albino *O. piceae*, Albino *O. pluriannulatum*	*O. floccosum*, *O. querci*	*Rhizopus* sp., *Trichoderma* sp., *Penicillium* sp.
Treatment K - OPC422 F93 3410	Albino *O. floccosum*, Albino *O. piceae*, Albino *O. pluriannulatum*	*Ophiostoma* sp. with black synnema, *O. floccosum*	*Trichoderma* sp., *Rhizopus* sp.,
Treatment M - OPC 703 F80 7073	Albino *O. floccosum*, Albino *O. piceae*, Albino *O. pluriannulatum*	*Ophiostoma* sp. with black synnema	*Trichoderma* sp., *Verticillium* sp.,
Treatment S - OPC 703 F93 7073	Albino *O. floccosum*, Albino *O. piceae*, Albino *O. pluriannulatum*	*Ophiostoma* sp. with black synnema, *S. sapinea*	*Verticillium* sp., *Rhizopus* sp., *Trichoderma* sp., *Penicillium* sp.
Treatment T - OPC 703 F80 3410	Albino *O. piceae*, Albino *O. pluriannulatum*	*Ophiostoma* sp. with black synnema, *O. floccosum*	*Trichoderma* sp., *Rhizopus* sp.
Treatment X – OPC 703 F80 3410	Albino *O. floccosum*, Albino *O. piceae*, Albino *O. pluriannulatum*	*Ophiostoma* sp. with black synnema, *O. querci*, *O. floccosum*, *S. sapinea*	*Trichoderma* sp., *Penicillium* sp., *Rhizopus* sp., *Verticillium* sp.
Treatment Y – 3410 freeze dried	Albino *O. pluriannulatum*	*Ophiostoma* sp. with black synnema	*Trichoderma* sp., *Rhizopus* sp., *Penicillium* sp.

6.5.8 Biological Control Field Trial IV (Summer 2000/2001)

As Treatment X in Biological Control Field Trial IV performed better than the other combinations of albino *Ophiostoma* species, two of the albino species within this mixture (OPC 703 and 3410) were chosen for a further field trial in the summer of 2000/2001. Another albino *O. pluriannulatum* strain 5040 was also chosen as this isolate performed well in previous trials. Samples were taken from the trial logs prior to inoculation at the establishment of the trial. A total of 38 samples were taken from the logs and two isolates of *Ophiostoma* species and 12 isolates of *S. sapinea* were cultured. Other fungi isolated included: *Trichoderma* species, *Penicillium* species, *Cladosporium* species, *Pestalotia* species, *Alternaria* species, *Epicoccum* species and *Verticillium* species.

After five months, logs inoculated with treatments consisting of albino *Ophiostoma* strains and controls (only water) were assessed for visual sapstain development and colonisation by albino *Ophiostoma* species, sapstain fungi and other wood inhabiting fungi. The presence of the native New Zealand bark beetle *Pachycotes peregrinus* was noted in the majority of logs in this field trial. The amount of coverage of visual sapstain over the trial as a whole ranged from 8% to 83% for a log treated with albino *O. piceae* strain OPC 703 and a log treated with a mixture of albino *O. floccosum* strains F13 and F40, respectively. There was significant log-to-log variation within treatments. The biggest variation for one treatment (albino *O. floccosum* strains F13 and F40) ranged from 14% to 83% in visual stain coverage. Log-to-log variation could be attributed to nutrient differences between trees or susceptibility of the wood to fungal colonisation.

The mean percent stain coverage for each treatment is displayed in Figure 6.12. There was a significant difference between the treatments for this trial (F=3.4, P=0.010). The means were compared using Tukey's test to find the significant differences. Treatments 2 and 4 (albino *O. piceae* OPC 703 and albino *O. pluriannulatum* 3410, respectively) were significantly different from the logs treated with the mixture of albino *O. floccosum* F13 and F40. All of the other means were not significantly different (P=0.05).

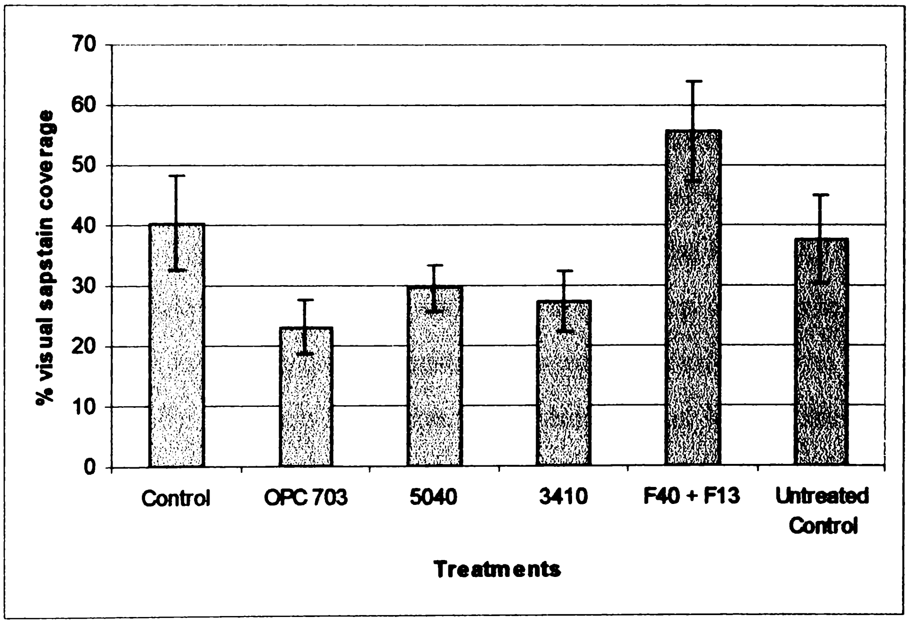


Figure 6.12: Visual sapstain coverage of sapwood after five months inoculation with various albino strains in Biological Control Field Trial IV.

Figure 6.13 shows the stain intensity of each treatment. There was no statistical difference between stain intensity and treatment for Biological Control Field Trial IV

(F=0.68, P=0.621). All the treatments had an average stain intensity that was between 3.8 and 4.2 (dark grey).

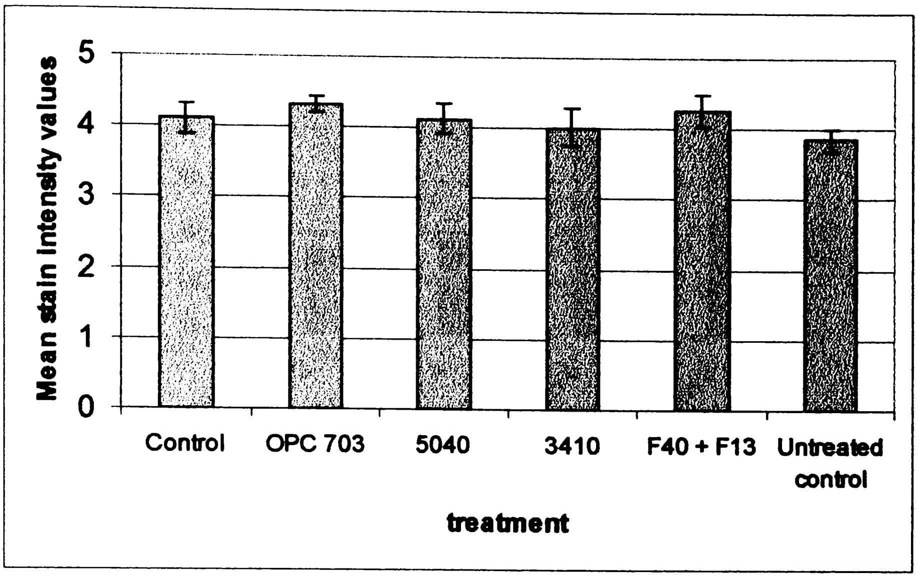


Figure 6.13: The mean stain intensity values after five months inoculation with various albino strains in Biological Control Field Trial IV.

The visual stain coverage data was compared according to the position within the log. The logs were sliced into four pieces and each cut face was assessed. The three cut faces were at the following measurements: 37.5cm (Position 1), 75cm (Position 2) and 112.5cm (Position 3). There was no statistical difference between the amount of visual stain in the middle of the log compared to each end of the log (F=0.03, P= 0.971), indicating no within log variation in the coverage of visual stain. The logs for this trial were placed in piles according to treatment, as there was limited space at the site. The position within the pile was compared to the amount of visual sapstain assessed (Figure 6.15). The Positions 1 to 4 were on the bottom of the pile, the Positions 5 to 7 were the second level of the pile and Positions 8 and 9 were at the top of the pile. There was a significant difference in the amount of sapstain coverage according to the position in the pile (F=4.27, P<0.000). The top logs were more stained than logs in the bottom of the pile.

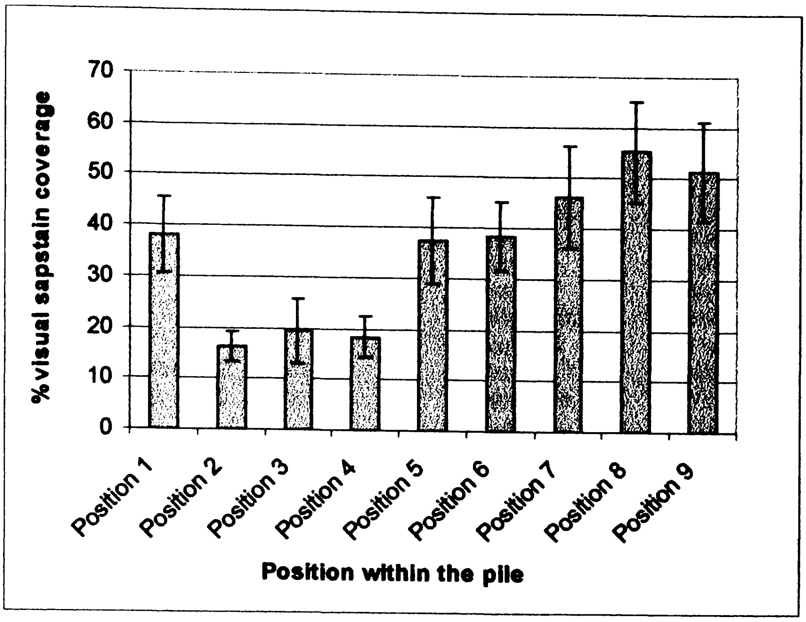


Figure 6.14: Percent visual sapstain coverage according to position within the pile for Biological Control Field Trial IV.

The total diameter and heartwood diameter of each log was measured. The amount of visual stain coverage was correlated to the total diameter of each log. There was no relationship between the diameter of the log and the percent visual stain coverage for Biological control Field Trial 1 (R^2=0.0003). The percentage sapwood in each log face was calculated from the total diameter and heartwood diameter measurements and compared with the percent visual stain coverage. There was no correlation between these factors (R^2= 0.1168).

Table 6.10 shows the fungi colonising the log in Biological Control Field Trial IV from culturing after five months in the field. The albino *O. floccosum* strains aggressively colonised the logs treated with these fungal strains but also colonised the other treatments. These fungi showed excellent colonisation but also an increased amount of stain coverage occurred for this treatment and will be discussed later. The albino *O. pluriannulatum* strain 3410 was not recovered from the logs treated with this fungus after five months in the field. The dosages of albino fungi inoculum were reduced for this trial and freeze dried product was used. The albino *O. piceae* strain successfully colonised the logs as well as showing a statistical decrease in the amount of stain coverage compared to the controls in this trial. The sapstain fungi found in this trial were some of the most commonly found fungi in New Zealand according to the survey of Farrell *et al* (1998) and data presented in Chapter 3. No differences were evident in the sapstain fungi colonising the different treatments.

Table 6.9: Albino *Ophiostoma* species, sapstain species and other fungi isolated and identified from Biological Control Field Trial IV.

Treatment	**Albino fungi**	**Sapstain fungi**	**Other fungi**
Control	Albino *O. floccosum*	*O. setosum,* *O. querci,* *O. piceae,* *O. huntii,* *O. piliferum,* *S. sapinea*	*Alternaria* sp., *Trichoderma* sp., *Rhizopus* sp., *Verticillium* sp., *Fusarium* sp., *Pestalotia* sp.
Albino *O. piceae* strain OPC 703	Albino *O. piceae,* Albino *O. floccosum*	*O. huntii,* *O. piliferum,* *O. querci,* *L. procerum,* *O. setosum,* *S. sapinea*	*Alternaria* sp., *Trichoderma* sp., *Rhizopus* sp., *Pestalotia* sp., *Fusarium* sp., *Penicillium* sp.,
Albino *O. pluriannulatum* strain 5040	Albino *O. pluriannulatum,* Albino *O. floccosum,*	*O. huntii,* *O. querci,* *O. setosum,* *O. piceae,* *O. floccosum,* *O. piliferum,* *S. sapinea*	*Rhizopus* sp., *Trichoderma* sp., *Penicillium* sp., *Fusarium* sp., *Pestalotia* sp., *Verticillium* sp.
Albino *O. pluriannulatum* strain 3410	Albino *O. floccosum*	*O. querci,* *O. piliferum,* *O. piceae,* *O. huntii,* *O. setosum,* *O. floccosum,* *S. sapinea*	*Verticillium* sp., *Rhizopus* sp., *Trichoderma* sp., *Fusarium* sp., *Alternaria* sp.
Albino *O. floccosum* Strains F40 and F13	Albino *O. floccosum*	*O. floccosum,* *O. huntii,* *L. procerum,* *O. piceae,* *O. querci,* *S. sapinea,* *O. setosum*	*Fusarium* sp., *Trichoderma* sp., *Rhizopus* sp., *Pestalotia* sp., *Alternaria* sp.
Untreated control	Albino *O. floccosum*	*O. huntii,* *O. querci,* *O. piceae,* *O. setosum,* *O. floccosum,* *S. sapinea,* *O. piliferum*	*Penicillium* sp., *Trichoderma* sp., *Geotrichium* sp., *Alternaria* sp., *Rhizopus* sp., *Fusarium* sp., *Verticillium* sp.

The mean percent colonisation of wild type sapstain fungi cultured from each treatment was calculated and shown in Figure 6.15. A total of 15 wood samples were taken from each log at the completion of the trial from the outer sapwood and inner sapwood on stained and unstained wood. The amount of sapstain fungi isolated from each log was recorded and then divided by 15 to calculate the percent colonisation of each log. There was a significant difference between the treatments and the amount of colonisation by wild type sapstain fungi (F= 3.11, P=0.017). The logs treated with the mixture of albino *O. floccosum* F13 and F40 had significantly less stain than the other treatments. There were no differences between the other treatments in the amount of wild type sapstain fungi colonising the wood.

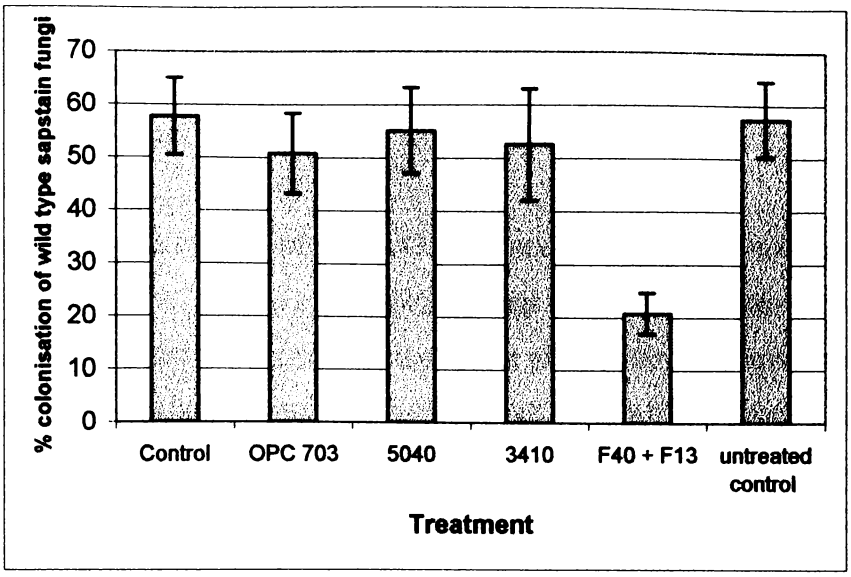


Figure 6.15: Mean percentage of colonisation of wild type sapstain fungi for each treatment in Biological Control Field Trial IV.

Not all albino strains were effective at preventing sapstain. This was an unexpected and unobvious result as previously it was considered that any albino or almost albino fungus would constitute a biological control effect on wood and prevent sapstain fungi from discolouring wood. Incomplete protection from sapstain by albino strains in Biological Control Field Trials III and IV suggests that the albinos were incapable of completely inhibiting growth by the target stain fungus. It is unclear whether this incomplete protection reflects an inability of albino to completely colonise the substrate or overgrowth of the stain fungus on the colonised wood. Blanchette *et al* (1994) found that Cartapip 97, had decreased colonisation 58% after 24 and 52 weeks in the field trials compared to colonisation of 95% after 8 weeks. Decrease in frequency of isolation of albino after five months may be a result of natural senescence or successful colonisation of wood by secondary fungi.

Biological control of sapstain over a four-month period is also an exceedingly long time for the albino strains to remain efficacious. Previous investigations indicate that albino strains performed well as biological control agents over a shorter period in field studies (Behrendt *et al*, 1995a, b). Logs used in New Zealand field trials were completely debarked, which resulted in the entire circumference of the logs being exposed. This large area of exposed sapwood and inadequate inoculum coverage may have contributed to a reduced effectiveness of sapstain control. Improved methods of inoculation and application could provide better coverage and better adherence to the logs. When using biological control agents, the forest industry may have to accept shorter periods of log storage (2-3 months instead of 6) and more rapid processing of logs treated with biological control fungi.

The treatment with the mixture of albino *O. floccosum* F13 and F40 was the most stained but had the least amount of wild type sapstain fungi isolated. The stain on this treatment is most likely associated with pigmentation of the albino strains themselves. These two-albino strains still produce pigmented synnema but have white hyphae when grown on synthetic media *in vitro*. It was evident throughout the research presented in this chapter that the albino *O. floccosum* strains F13 and F40 produced pigmented hyphae in association with temperature, and in two field trials established in summer, Albino Colonisation Field Trial I and Biological Control Field Trial IV. These fungi are successful at colonisation of *P. radiata* in both the laboratory and field trials, but would not be good candidates for further biological control studies. These strains were used for successful biopitch field trials in a pulp mill in South Australia, where they colonised the wood and reduced the amount of extractives in *P. radiata* wood chips (Farrell *et al*, 2000).

The log diameters and total percentage of sapwood varied in Biological Control Field Trial III and IV and therefore the logs were assessed with regard to these parameters and examined for potential compounding effects of sapstain coverage. Log diameter or the percentage sapwood had no influence on the level of discoloration. Yang *et al* (1999) found that sapstain fungi affected smaller logs more than larger logs and that there was more variation of stain severity in smaller logs. The variation in these trials was attributed to the proportion of sapwood in each log face as well as the susceptibility of individual trees to sapstain fungi (Yang et al, 1999).

Contamination of the control logs with albino strains occurred in both Biological Field Trial III and IV. Behrendt et al (1995a,b) also noted the presence of the albino *O. piliferum* strain Cartapip 97 on control logs in biological field trials. Albino growth in the control logs most likely resulted from rain and wind dispersal of spores from treated to untreated logs, inadvertent contact during inoculation or possible animal or insect vectoring. The spores of *Ophiostoma* species are easily dispersed by rain splash, wind, insects, animal vectors or during harvest and processing operations (Dowding, 1970; Dowding, 1973).

The bark beetle *Pachycotes peregrinus* was isolated from logs in Biological Field Trial IV. The beetle is a borer in moist logs and of economic importance in New Zealand as it attacks logs stockpiled in the forest near breeding sites (Bain, 1977). This beetle could have vectored wild type sapstain fungi to the logs.

6.5.9 *In vitro* interactions of Albino *Ophiostoma* strains and other wood inhabiting fungi including sapstain species

The interactions of albino *Ophiostoma* strains and wild type sapstain fungi on synthetic media are shown in Table 6.10. Zonal barriers formed by albino *O. floccosum* strains were evident when inoculated with *S. sapinea* (Table 6.10 and Figure 6.16). In most cases the albino *O. pluriannulatum* and *O. piceae* cultures were overgrown by *S. sapinea* on agar media (Table 6.10 and Figure 6.16). The interactions between wild type *Ophiostoma* species and albino *Ophiostoma* strains were also compared. In all cases the fungi interacted when they reached each other and eventually intermingled together (Table 6.10 and Figure 6.16).

Table 6.10: Interactions of albino strains and wild type sapstain fungi on synthetic media.

	Interaction of albino in response to competing fungi			
	Sapstain fungi			
Albino *Ophiostoma* strain	***S. sapinea***	***O. ips***	***O. querci***	***L. procerum***
O. floccosum				
F13	Zonal barrier	Two fungi interacted	Two fungi interacted	Two fungi interacted
F40	Zonal barrier	Two fungi interacted	Two fungi interacted	Two fungi interacted
F80	Zonal barrier	Two fungi interacted	Two fungi interacted	Two fungi interacted
F93	Zonal barrier	Not tested	Two fungi interacted	Two fungi interacted
F124	Zonal barrier	Two fungi interacted	Two fungi interacted	Two fungi interacted
F132	Zonal barrier	Two fungi interacted	Two fungi interacted	Two fungi interacted
F145	Zonal barrier	Two fungi interacted	Two fungi interacted	Two fungi interacted
O. piceae				
OPC 422	Overgrowth by *S. sapinea*	Not tested	Not tested	Not tested
OPC 703	Zonal barrier	Not tested	Not tested	Not tested
OPC 846	Overgrowth by *S. sapinea*	Not tested	Not tested	Not tested
OPC 848	Overgrowth by *S. sapinea*	Not tested	Not tested	Not tested
OPC 849	Overgrowth by *S. sapinea*	Not tested	Not tested	Not tested
O. pluriannulatum				
3410	Overgrowth by *S. sapinea*	Two fungi interacted	Two fungi interacted	Two fungi interacted
7073	Overgrowth by *S. sapinea*	Not tested	Not tested	Not tested
5040	Overgrowth by *S. sapinea*	Not tested	Not tested	Not tested

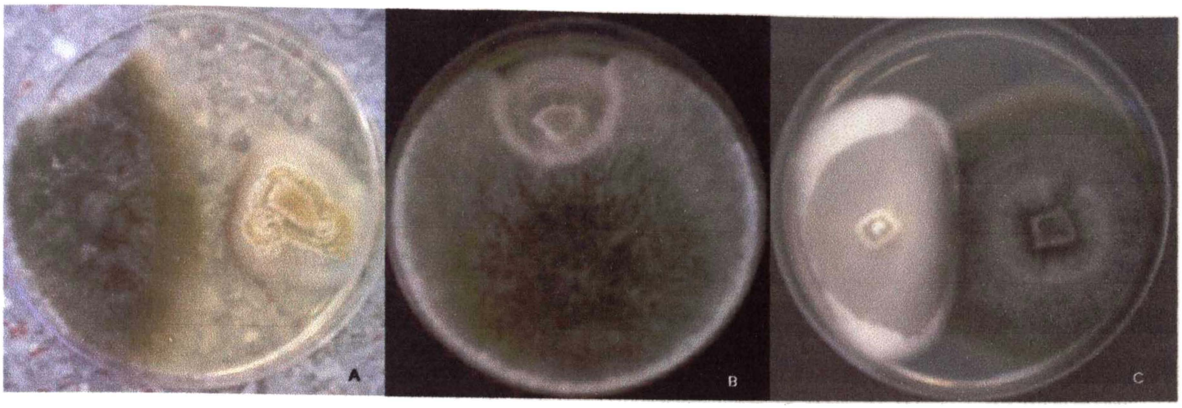


Figure 6.16: Interaction of albino Ophiostoma strains with sapstain fungi. A: Zonal barrier formed between *S. sapinea* (isolate 35) and albino *O. floccosum* strain B13. B: *S. sapinea* (isolate 35 overgrowing albino *O. pluriannulatum* strain 3410. C: *L. procerum* (isolate 1852) and albino *O. pluriannulatum* 3410 growing together.

The interactions of albino *Ophiostoma* strains and other wood inhabiting fungi on synthetic media are shown in Table 6.11. Zonal barriers formed by albino *O. floccosum* strains were evident when inoculated with *E. nigrum* (Table 6.11 and Figure 6.17). The interactions between *A. alternata*, *C. cladosporioides* and albino *Ophiostoma* strains were also compared. In all cases the fungi interacted when they reached each other and eventually intermingled together (Table 6.11 and Figure 6.17).

Table 6.11: Interaction of albino strains and other wood inhabiting fungi on synthetic media.

	Interaction of albino in response to competing fungi		
	Wood inhabiting fungi		
Albino *Ophiostoma* strain	***C. cladosporioides***	***E. nigrum***	***A. alternate***
O. floccosum			
F13	Two fungi interacted	Zonal barrier	Two fungi interacted
F40	Two fungi interacted	Zonal barrier	Two fungi interacted
F80	Two fungi interacted	Overgrowth by *E. nigrum*	Two fungi interacted
F93	Two fungi interacted	Zonal barrier	Two fungi interacted
F124	Two fungi interacted	Overgrowth by *E. nigrum*	Two fungi interacted
F132	Two fungi interacted	Not tested	Two fungi interacted
F145	Two fungi interacted	Overgrowth by *E. nigrum*	Two fungi interacted
O. piceae			
OPC 422	Two fungi interacted	Overgrowth by *E. nigrum*	Two fungi interacted
OPC 703	Two fungi interacted	Overgrowth by *E. nigrum*	Two fungi interacted
OPC 846	Two fungi interacted	Overgrowth by *E. nigrum*	Two fungi interacted
OPC 848	Two fungi interacted	Overgrowth by *E. nigrum*	Two fungi interacted
OPC 849	Two fungi interacted	Overgrowth by *E. nigrum*	Two fungi interacted
O. pluriannulatum			
3410	Two fungi interacted	Overgrowth by *E. nigrum*	Two fungi interacted
7073	Two fungi interacted	Overgrowth by *E. nigrum*	Two fungi interacted

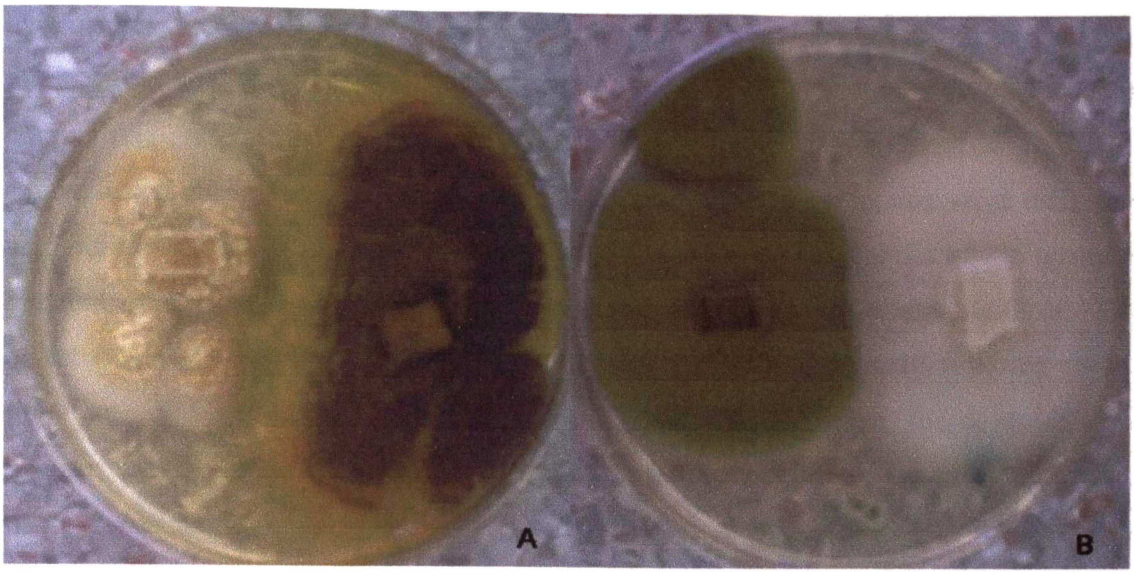


Figure 6.17: Interaction between albino *Ophiostoma* strains and wood inhabiting fungi. A: Zonal barrier formed between albino *O. floccosum* F13 and E. nigrum B: *C. cladosporioides* and albino *O. pluriannulatum* 3410 growing together.

Some albino *Ophiostoma* strains induced pigmentation in response to challenging fungi, either *S. sapinea*, wild type *Ophiostoma* species or wood inhabiting fungi (Table 6.12, Figure 6.16A and Figure 6.17A).

Table 6.12: Pigment formation of albino strain in responses to a competing fungus on synthetic media.

	Pigment production of albino in response to competing fungi						
	Sapstain fungi and wood inhabiting fungi						
Albino *Ophiostoma* strain	***S. sapinea***	***E. nigrum***	***A. alternata***	***C. cladosporioides***	***O. ips***	***O. querci***	***L. procerum***
O. floccosum							
F13	Yes	Yes	Yes	No	No	No	Yes
F40	Yes	Yes	Yes	No	No	No	Yes
F80	No	No	Yes	No	Yes	Yes	No
F93	Yes	Yes	Yes	Yes	Not tested	No	No
F124	Yes	No	Yes	No	No	No	No
F132	Yes	Not tested	Yes	No	Yes	No	Yes
F145	Yes	No	Yes	Yes	Yes	No	Yes
O. piceae							
OPC 422	Yes	No	Yes	No	Not tested	Not tested	Not tested
OPC 703	No	No	Yes	No	Not tested	Not tested	Not tested
OPC 846	No	No	No	No	Not tested	Not tested	Not tested
OPC 848	No	No	Yes	No	Not tested	Not tested	Not tested
OPC 849	No	No	No	No	Not tested	Not tested	Not tested
O. pluriannulatum							
3410	No	No	No	No	No	No	No
7073	No	No	No	No	Not tested	Not tested	Not tested
5040	No	Not tested	Not tested	Not tested	Not tested	Not tested	Not tested

Neutralistic or mutualistic interactions were observed with most pairing of albino *Ophiostoma* species and wild type sapstain fungi or other wood inhabiting fungi. The mode of biocontrol action of most of the albino fungi was therefore considered to be in competition and in nutrient capture. However, in these assays, albino *O. floccosum* strains were able to stop the growth of *S. sapinea* by the formation of a diffusible metabolite (zonal barrier formation) into the media. Chemical analysis of the potential metabolite would be required to successfully confirm that it was a true antibiotic. The effect of an *Ophiostoma* species on *S. sapinea* was not previously reported in the literature. When grown in dual culture with albino *O. floccosum*, the colonies of *S. sapinea* were initially inhibited and ceased to grow long before contact with the hyphae of *O. floccosum*. Therefore, the antifungal activity is due extensively to its antifungal compounds rather than any parasitic function. Benko and Highley (1990) found that formation of inhibition zones varied greatly on different media, probably because of variable production of inhibitory metabolites. Payne and Bruce (1999) found that malt yeast extract agar was the best medium in terms of developing inhibition zones. Fungal colonises often produce pigment when opposing another fungus on agar medium. Henson *et al* (1999) stated whether this is an offensive or defensive response or both is not known because melanin can be toxic and protective.

When two cultures are in "deadlock", neither species is capable of dominance over the other and a thin zone of uncolonised agar is evident between the fungal colonies. On agar plates a region of dense mycelial growth, aerial hyphae or pigment production frequently occurs at the edge of the colony (Owens *et al*, 1994). In the interaction experiment described in this chapter some albino strains produced a pigment at the edge of the colony. Gibbs (1993) described pigment production in *Ophiostoma* species as occurring in response to wounding and or stress and may enable the fungi to with stand lytic enzymes. The reversion of the albino strains in response to competitive stress was unexpected. One explanation of this phenomenon may be the production and secretion of metabolites of the melanin production pathway by the competiting fungi into the medium and the albino is then able to revert with the presence of missing metabolites. Zimmerman *et al* (1995) found that melanin production was restored when mycelia of the albino strain was grown on media supplemented with an extract of spent culture fluid derived from a pigmented strain of *O. piliferum*. The extract contained scylatone and intermediate of the pentaketide pathway. However, if this were the case one would expect that all the albino strains revert in the presence of another fungus. It is unsure if all of the albino strains used in this chapter were "true" albinos. It was clear that isolates of albino *O. floccosum* and *O. piceae* are capable of melanin production under certain conditions. In development of the albino strains used cultures of *O. floccosum* with varying melanin production were described. The mechanism of melanin formation of these albinos are not completely known, this study suggests that different genetic factors are likely to be responsible for the overall pigment production. Pigment production in the vegetative mycelium and pigments produced in and around synnema required different mating selections to obtain melanin free cultures (Held et al, in press).

6.5.10 Summary

This study provided results of four field trials demonstrating that treating logs with specific albino *Ophiostoma* strains could reduce the appearance of stain and colonisation by sapstain fungi. Each trial differed in either the delivery of the fungal inoculum, the amount of inoculum or the inoculum strains used whether a mixture or single strains. If biological control is to be considered a viable option for the forest industry it is necessary to investigate aspects such as formulation and efficiency of the delivery system (Brown and Bruce, 1999). In advancing albino technology to the commercialisation of a product, methods of application of the fungus needs to be addressed. Ideally the application method of a control agent should be compatible with the existing procedures of harvesting and processing. In Biological Control Field Trial II, albino strains were applied successfully to the logs using an industrial spray system utilised for anti-sapstain chemical treatments. This system was successful at applying the spore suspension to all surfaces of the logs. The albino strains used in this thesis were applied a day after harvesting and debarking. Work with Cartapip 97 showed that the biological control fungus must be applied as soon as possible for effective control (Blanchette *et al*, 1994). If other organisms have sufficient time to colonise the sapwood subsequent treatment with an albino isolate did not help to eradicate the established sapstain fungi. In a commercial environment the movement of logs to a processing plant where the biological control agent is applied may take longer than one day. Other options of application of the control agent must be considered.

Schoeman *et al* (1994) studied the application of spores of a biological control agent *Trichoderma harzianum* applied in the oil of chain saws to freshly felled pine logs to reduce deterioration. *T. harzianum* spores were present in the chainsaw oil and they transferred successfully to the wood surface during sawing. Colonisation of this fungus was abundant on the billets. Schoeman *et al* (1994) noted that these observations indicated that the film of oil delivering the conidia to the woody substratum actually increased the survival of the spores.

Tucker *et al* (1997) used a bench-top pressure impregnation system to apply spores of a *Trichoderma* species to wood blocks. This study by Tucker *et al* (1997) established that a pilot preservation plant could be used to impregnate wood samples with a fungal spore suspension for biological control purposes. Spores retained viability following pressure treatment but the exact nature of the uptake and penetration of spores was not determined (Tucker *et al*, 1997).

Farrell *et al* (2000) developed guidelines for successful inoculation of albino *Ophiostoma* strains onto wood chips for biopulping, which included producing a fungal inoculum from growth media, which allowed it to be freeze dried with viability (shelf life) for over six months. The spores were sprayed at a dosage rate of fifty tonne of wood sprayed with 2 cubic metres water/fungi at a 10^6 –10^7 colony forming units of fungi per millilitre suspension; thus, each square millimetre of surface is sprayed with 0.725 to 7.25 fungal cells (Farrell *et al*, 2000). The field trials in this thesis research varied in the inoculum dosage and both freeze dried fungal products and fresh spores were used. The more successful trials, Biological Control Field Trial I and II, both used higher dosages of colony forming units (1 X10^8 and 1 X 10^9 respectively) than Biological Control Field Trial III and IV (1 X 10^7 and 1 X 10^6 respectively). Biological control of solid wood, therefore, required a high dosage of colony forming units. A controlled experiment using differing dosages of an albino fungus would be needed to confirm this point. Biological control using competition via primary resource capture is dependent on spores being applied to the wood, germinating and colonising the wood. There must be a balance between too many fungal spores competing for the same niche and not enough spores to successfully cover the entire exposed circumference of the log. Holmer and Stenlid (1993) studied the inoculum size for the competitive ability of wood decomposing fungi and found that competitive success was determined by the amount of mycelium, the greater the mycelium the more success. Payne and Bruce (2001) described that significant reduction in sapstain of nonsterile wood was demonstrated at an application of 9 X 10^6 to 9 X 10^8 colony forming units per cm^2. This is significantly more than the application rates described in this chapter for successful biological control of sapstain.

Kang and Morrell (2000) raised concerns that biological control using one organism may not be sufficient to control many different species of sapstain organisms. Results presented in this chapter showed that single strains of albino species could control several different genera of stain fungi commonly found in New Zealand. The use of mixtures of multiple albino strains to control sapstain in the field was also investigated. Application of multiple biological control agents was possible and after five month in the field each strain with in a mixture was isolated in most of the treatments. Albino *O. piceae*, *O. floccosum* and *O. pluriannulatum* strains were all able to successfully colonise wood when inoculated together. Even though Biological Control Field Trial III showed no statistical difference between the treatments and the controls, one treatment (Treatment X) had less stain than all of the other treatments.

Research into strategies that could improve the performance of biological control agents in the field is needed. The use of compatible anti-sapstain chemical and biological control agents in order to have synergistic or augmentative control were explored to a limited extent. Combined chemical and biological control could improve the performance of the biological control agent. It would require that either the biological control agent is resistant or tolerant to the chemical agent, or that they are applied sequentially, with the biological control agent penetrating into sapwood ahead of the chemical agent's topical application. Interesting research was shown using this approach with essential oils or biocides and biological control agents (McAfee and Gignac, 1997; Dawson-Andoh *et al* 2000).

Another approach to aid colonisation of the biological control agent on wood could be to alter the wood substrate to stimulate spore germination or to limit colonisation of competing fungi (Dawson-Andoh and Lovell, 2000). Dawson-Andoh and Lovell (2000) studied sugar alcohols, inorganic and amino acids for their ability to selectively enhance germination of spores of the biological control agent *Gliocladium roseum*. Some amino acids selectively enhanced germination of *G. roseum* over the sapstain fungus *O. piceae*.

Melanin is not a required colonisation factor for sapstain fungi, as the albino fungi lacking melanin remain capable of colonisation of the wood substrate. This phenomenon is also evident for virulence factors for albino mutants of other fungi (Frederick *et al*, 1999). Research on other fungi showed some albino mutants are disadvantaged in the wild without the protective effect of melanin. Albino mutants of the *A. alternata* produce lesions on Japanese pears, but their conidia are more sensitive to UV (Kawamura *et al*, 1999).

The albino fungi presented in this chapter varied in their growth characteristics and in their ability of grow at 15, 23 and 30°C on both synthetic media and on *P. radiata* specimens. The albino fungi were able to grow as well or better than the wild type strains in synthetic media as well as on *P. radiata* specimens in the laboratory or in the field environment. This chapter provided results from four field trials showing variation in the biological control ability of the albino strains against wild type sapstain fungi. The mode of action of the albino strains mainly through competition and the use of primary resource capture. Albino *O. floccosum* strains were shown however to produce an antibiotic affect when challenged with *S. sapinea.*

The results presented demonstrate that biological control using albino stains of *Ophiostoma* can be successful in New Zealand but more research investigation is needed to optimise these new biological control processes. A large number of albino strains of *O. piceae*, *O. pluriannulatum* and *O. floccosum* are available from this study and can be used in continued field evaluations in New Zealand to control sapstain in *P. radiata* logs and for other bioprocess technologies.

7 Development of a detection method for sapstain fungi using proteomics

7.1 Introduction

The ability to detect sapstain fungi in wood before substantial damage has occurred and to differentiate sapstain fungi from common surface moulds is an important goal in wood protection. The traditional methods for detecting fungi in wood are by direct microscopy and isolation of organisms into pure culture. Direct microscopy demonstrates the presence of fungi, but rarely allows for the identification of fungal species. Isolation into pure culture is only the first step for the identification of species as mating capability studies and DNA sequence analysis are often required. The methods for unequivocal species identification are time consuming and may take up to six weeks.

A technique is investigated in this chapter that could potentially assist in the identification of sapstain fungi or assist in the differentiation between sapstain fungi and other wood inhabiting fungi. The technique is to identify unique protein spots in two-dimensional gel using proteomics. While two-dimensional gel electrophoresis and proteomics are widely used to study the proteome of many organisms, there are only a few reports of the use of these techniques to study fungi (Lim *et al*, 2001; Nandakumar and Marten, 2002).

This chapter will firstly discuss the literature available on the identification of sapstain fungi, which is currently used including morphological and physiological features, DNA analysis and mating capabilities. The literature review also includes methods (antibody development and isozyme analysis) that have been developed for detection of sapstain fungi.

7.2 Hypothesis, Aims and Objectives

The hypothesis was that specific proteins from the cell wall of sapstain fungi would be found that could be further developed into an antibody-based diagnostic tool. The main aim was to determine if there were unique proteins in each of the sapstain species tested that could potentially be used for the development of an antibody based diagnostic method. The main objective was to explore the use of two-dimensional gel electrophoresis and proteomic analysis to detect unique cell wall proteins that could be further developed as diagnostic tools.

7.3 Literature Review

7.3.1 Methods of identification and detection of sapstain fungi

Methods for the identification of sapstain fungi, especially *Ophiostoma* species, currently use a holistic approach with a combination of techniques utilised, including phenotypes (morphological and physiological), mating reactions and phylogenetic analysis to obtain a species identification (Harrington *et al*, 2001). All of these techniques were utilised in the identification of *Ophiostoma* species within this thesis research.

7.3.1.1 Phenotypes

The taxonomy of *Ophiostoma* species and *S. sapinea* was reviewed in Chapter 1 (Sections 1.4.3.2.2 and 1.4.3.2.1 respectively). Physiological and morphological characters were found to be useful in identifying cultures to species level (Harrington *et al*, 2001). Morphological identification utilised the formation of different anamorphs in culture, as well as, distinct growth characteristics on agar media. *Ophiostoma* species with similar anamorphs tend to be morphologically very similar and exhibit no inhibition when growing together on wood or in culture, these conditions tend to promote misidentification when morphology alone was used (Harrington *et al*, 2001).

7.3.1.2 Phylogenetic analysis

Molecular analysis based on DNA sequences was recognised as a reliable method to reveal genetic relationships and can be used to evaluate the relationships of fungi at any taxonomic rank (Gardes and Burns, 1993). DNA based methods require the location of targeted DNA giving a high degree of discrimination. The fungal ribosomal RNA (rRNA) is frequently used as it contains highly conserved and variable regions, and is found in multiple copies in the genome (Gardes and Burns, 1993). The variable internal transcribed spacer-derived-specific (ITS) region of rDNA genes are also extensively studied and used to differentiate taxonomic entities at the species level. Restriction fragment length polymorphisms (RFLPs) and random amplified polymorphic DNA (RAPD) markers are widely used to study molecular variation with fungal populations

(Pipe *et al*, 1995). The ITS region, along with RFLPs and RAPDs within this region, were used to identify species of *Ophiostoma* and *Botryosphaeria* (Hausner *et al*, 1993; Pipe *et al*, 1995; Jacobs and Rehner, 1998; Kim *et al*, 1999; Harrington *et al*, 2001; Jacobs *et al*, 2001; Zhou and Stanosz, 2001). Harrington *et al* (2001) identified restriction enzymes that would differentiate among *O. piceae*, *O. floccosum*, *O. querci* and *O. setosum* (Table 7.1). These species could be differentiated based on HaeII digestion. *O. setosum* and *O. piceae* have the same restriction pattern with HaeII, but these two species can be differentiated based on DdeI restriction fragments (Table 7.1).

Table 7.1: Restriction fragments of the amplified ITS product from species in the *Ophiostoma piceae* complex isolated in New Zealand (source Harrington *et al*, 2001).

Species	HaeII fragments (bp)	DdeI fragments (bp)
O. piceae	400, 200, 120	290, 270, 190
O. floccosum	280, 200, (120)[a]	290, 270, 190
O. setosum	400, 200, 120	270, (190)[a], 100
O. querci	520, 200	290, 270, 190

[a] Band sizes in parentheses are for two co-migrating bands

With experience, it is possible to amplify the ITS region using scrapes of mycelium for template DNA. Kim *et al* (1999) showed that the ITS region of *O. piceae* and *O. querci* can also be amplified directly from the conidia at the apex of synnemata. Without the need for DNA extraction, the PCR-RFLP technique could be completed in a single day (Harrington *et al*, 2001). The cost involved in phylogenetic analysis limits the regular use of these techniques.

7.3.1.3 Immunological methods

Immunological methods primarily ELISA (enzyme-linked immunosorbent assays) were used successfully for detecting plant pathogens including fungal species (Wycoff *et al*, 1987; Dewey *et al*, 1989; Plascencia *et al*, 1996) in herbaceous plants. Breuil *et al* (1988 and 1990) developed an ELISA technique to detect an unidentified *Ophiostoma* species in artificially inoculated wood using polyclonal antibodies. These immunological assays were able to detect sapstain fungi in wood but were cross-reactive with other fungal species. Banerjee *et al* (1994) produced monoclonal antibodies to *O. piceae* that were less than 5% reactive with other fungi. Chemical and enzymatic modification of the antigen revealed that the epitopes recognised by the monoclonal antibodies were glycospecific.

Immunological methods were used to study various aspects of the pathogenicity of *O. ulmi* and *O. novo ulmi*, the Dutch elm disease fungi (Dewey and Brasier, 1988). Antibodies were also raised against several toxins implicated in the pathogenicity of *O. ulmi* and *O. novo ulmi* (Benhamou *et al*, 1985). These antibodies were used to demonstrate the distribution of toxins within infected plants (Benhamou *et al*, 1985).

Successful antibodies were raised against the various antigens from the fungi causing human sporotrichosis, *Sporothrix schenkii*. The various antigens included: L-rhamno-D-mannans (Lloyd and Travassas, 1975), glucoproteins (Kurata, 1981) and peptidorhamnomannans (Alves *et al*, 1994).

Taxonomic applications using immunological techniques are limited due to the large number of similar antigens shared by closely related species making detection of unique antigens difficult (Breuil and Seifert, 1993).

7.3.1.4 Isozyme analysis

As a source of non-morphological characters, enzyme electrophoresis is a widely used technique. Electrophoresis of soluble enzymes is an indirect method of determining genetic differences at enzyme loci, as well as, used to delineate taxa that are morphologically similar (Zambino and Harrington, 1989). The isozyme variation was shown to correspond with morphological variation and proved useful in showing relationships among isolates, species and species clusters of the genus *Leptographium* (Zambino and Harrington, 1989; Zambino and Harrington, 1992) and for the *Ceratocystis coerulescens* complex (Harrington *et al*, 1996).

7.3.2 Fungal cell walls

The fungal cell wall is the largest organelle of a filamentous fungus that performs a variety of functions and its composition, as well as, structure is constantly modified during growth and development. Fungal cell walls consist of a complex matrix of carbohydrate polymers and proteins. The cell wall plays a role in physical and osmotic protection and contributes in cell-to-cell interactions such as recognition and adhesion (Lim *et al*, 2001). The cell wall is also an essential structure required for maintenance of cell integrity and shape (Pardo *et al*, 2000). The cell wall exhibits a dynamic nature, undergoing profound changes during different cellular processes, namely budding, mating and dimorphic transition (Pardo *et al*, 2000).

The existence of glycoprotein complexes in the cell walls of filamentous fungi is well documented. D-glucose, D-mannose and N acetyl glucosamine are the most ubiquitous monosaccharides detected in most fungi (Bartnicki-Garcia, 1968; Wessels, 1993). Bartnicki-Garcia (1968) proposed that fungi could be subdivided into at least eight categories based on dual combinations of the principle polysaccharides of vegetative walls. The major wall components considered in this distribution were chitin, cellulose, chitosan, mannans, β1,3 and β1,6 glucans, and a glucogen-like polymer. According to this scheme, Oomycetes were classified as cellulose-glycan group while Ascomycetes, Basidomycetes and Deuteromcetes were considered chitin-glycan group. *Ophiostoma* species are an exception to this model with both cellulose and chitin being found in the cell walls of *O. ulmi* (Smith *et al*, 1967; Jewell, 1974; Benhamou, 1988). The cell walls of *Ophiostoma* species are also known to contain rhamnose, mannose and glucose (Spencer and Gorin, 1971; Pryzbyl and De Hoog, 1989).

The yeast *Saccharomyces cerevisiae* is the major experimental model to address cell wall structure and organisation of fungi (Pardo *et al*, 2000). The major components are β-glucans (formed by β1,3 and β1,6 linkages), mannoproteins and chitin. The β1,3 glucan and chitin are responsible for cell wall strength and the mannoproteins determine the porosity and acts as structural proteins or as enzymes involved in cell wall biogenesis.

The general method of studying fungal cell wall proteins was by the isolation and purification of individual proteins of interest (Lim *et al*, 2001). Recent advances in the field of proteomics made tools available to investigate whole organelles. The use of two-dimensional electrophoresis, availability of complete genome sequences and the development of mass spectrometry for peptide mass fingerprinting allowed for the complete identification of entire proteomes. Lim *et al* (2001) studied the cell envelope proteins of *Trichoderma reesei* using the application of proteome technology.

The fact that cell wall enzymes and structural proteins must be located at the cell surface makes protein an ideal target for identification purposes.

7.3.3 Introduction to proteomics/ electrophoresis analysis

Proteomics involves the combined applications of advanced separation techniques, mass spectrometry and bioinformatics tools to characterise proteins in complex biological mixtures. Two-dimensional gel electrophoresis for the separation of protein mixtures provides a powerful tool for surveying the biological complexity of a proteome (the protein complement expressed by a genome) at the molecular level (Wilkins *et al*, 1996). Peptide mass fingerprinting and automated protein database searching are commonly used for the high throughput identification and characterisation of proteins excised from two-dimensional gels (Wilkins *et al*, 1996).

Two-dimensional polyacrylamide gel electrophoresis was originally described by O'Farrell (1975) and allows for separation of proteins on the basis of the differences in their isoelectric points (p*I*) in the first dimension and molecular masses in the second dimension. The technology was improved with the introduction of commercially manufactured immobilised pH gradient (IPGs) that increase the reproducibility of this technique (Wilkins *et al*, 1996).

One advance in protein quantification was with the use of the new fluorescent stain, Sypro Ruby. Sypro Ruby stains are complexes of ruthenium (a luminescent transition metal) that binds avidly to proteins by a mechanism primarily involving lysine, arginine and histidine residues (Patton, 2000). This stain detection limits are similar to silver staining but the stains linear dynamic range is ten times greater than silver staining (Harry *et al*, 2000).

In order to identify individual proteins of the proteome separated by two-dimensional gel electrophoresis, a rapid means of screening large numbers of proteins was required (Wilkins *et al*, 1996). Individual proteins from gels were traditionally identified by immunoblotting, N-terminal microsequencing, internal peptide sequencing, comigration of purified proteins with known proteins or by the overexpression of homogenous genes of interest (Wilkins *et al*, 1996). These techniques are powerful identification tools, but they are too slow and expensive in screening thousands of proteins (Wilkins *et al*, 1996).

Mass spectroscopy is the technique of choice for the large-scale identification of two-dimensional gel separated proteins. Key proteins separated by two-dimensional gel electrophoresis are in gel digested with trypsin. Trypsin cleaves the protein backbone at the amino acids arginine and lysine. The molecular weights of the resulting tryptic peptides are measured using matrix assisted laser desorption/ionisation (MALDI) mass spectrometry (Pandey and Mann, 2000). MALDI is used typically in conjugation with time of flight (Tof) mass analysers, which produces information on the molecular weights of the digested proteins and peptides (Yates, 2000). The identity of an unknown protein is determined by comparing the molecular weight map of the unknown protein with the theoretical molecular weights of peptides generated from protein databases. This technique is commonly referred to as mass fingerprinting and a number of search programs are found on the World Wide Web (http://expasy.proteome.org.au) (Walsh *et al*, 1998). Peptide-mass searching is a powerful tool for rapid and sensitive protein identification, particularly in species for which the complete genome is sequenced.

7.4 Materials and Methods

7.4.1 Analysis of the cell wall proteins of *Ophiostoma* species and other wood fungi

Cell wall isolation and proteomic work was carried out at the Australian Proteome Analysis Facility, Macquire University, Sydney, Australia. The candidate visited the facility and assisted in the initial set up of the experiments. The staff at the facility carried out all other work. A peptide mass map for each spot analysed was obtained from the Australian Proteome Analysis Facility. These peptide mass maps were reanalysed by the candidate, using the protein databases prior to the completion of this thesis to reconfirm that successful identification of the protein spots was not possible. This was due to protein databases being updated constantly.

7.4.1.1 Fungal cultures

Four sapstain fungi (*L. procerum* strain 1852; *O. querci* strain 1688; *O. ips* strain P36; *S. sapinea* strain D4) and three wood inhabiting fungi (*Alternaria alternata*, *Trichoderma koningi* and *Epicoccum nigrum*) were selected for proteomic analysis. Each fungal culture was grown in one litre liquid malt yeast extract broth in a two-litre flask at 25°C in an incubator. The cultures were not shaken to encourage the formation of a higher content of mycelium. After seven days growth the fungus was harvested by centrifugation at 25, 482g (Beckman Model J2-21M Induction Drive Centrifuge with a JA-10 rotor) for 20 minutes and the resulting supernant removed. The pellet containing mycelium was resuspended in 100mM Tris-HCl pH 7 and placed in sterile containers, which were transported to the Australian Proteome Analysis Facility on ice.

7.4.1.2 Cell wall isolation

The cells were subjected to repeated rounds of high-pressure compression in a French Press (SCM Aminco, Urbana, Il, USA). Following this, the cells were disrupted by sonication (4 x 15 seconds) in the presence of glass beads. Breakage was determined at intervals by phase contrast microscopy. Following rupture, the cell walls were cleaned of cytosolic content in cell-breaking buffer (0.1M NaCl, 20mM Tris-HCl pH 7.0, 2mM EDTA, 0.1% SDS, 20μL protease inhibitor cocktail (0.2mM, EDTA 2mM, Leupeptin 20uM, Pepstatin 1uM)). The mycelial fragments were collected by gentle centrifugation (650g for 10min), twice with cell-breaking buffer and finally resuspended in cell breaking buffer. About 15mL of sample was layered on the top of 20mL of 30% sorbitol in cell-breaking buffer and centrifuged at 650g for 20 min. The pellet was washed with 30mL of cell-breaking buffer by resuspending and centrifuging at 650g. Finally, the isolated cell wall preparations were used for two-dimensional electrophoresis analysis.

7.4.1.3 Two-dimensional gel electrophoresis

Two millilitres of Multiple Surfactant Solution containing: 5M urea, 2M thiourea, 2mM tributyl phosphine, 2% 3-((3-cholamidopropyl) dimethylammonio)-1-propane-sulfonate (CHAPS), 2% sulfobetaine pH 3-10, 1% 3-10 carrier ampholytes, 40mM Tris (all from Bio-Rad, Richmond, CA, USA) was added to 100mg of dried cell wall. The mixture was subjected to bead beating in 2ml tubes containing 1mm glass beads. The remaining insoluble material was removed by centrifugation at 15 000g for 10 minutes and the supernant was collected. The supernant was stored at –20°C until required if not processed immediately.

For isoelectric focusing (IEF), precast 17cm long, ph 4-7 range immoblised pH gradient (IPG) strips (Bio-Rad) were used. Proteins were applied using sample cups. The dried IPG gels were assembled into a reswelling cassette and rehydrated overnight to their original thickness of 0.5mm in the combined cell wall protein and the multiple surfactant solution. IEF was carried out in a Multiphor II electrophoresis unit (Amersham Pharmacia, Uppsala, Sweden) and subjected to a gradient voltage application that amounted to 61.4kVh. After focusing, the gels were equilibrated for 20 minutes in 15ml of equilibration solution containing 6M Urea, 2% SDS, 3mM Tris-HCl, pH 8.8, 20% glycerol, 5mM tributyl phosphine, and 2.5 acrylamide. For the second dimension, 20cm

8-18% polyacrylamide gradient gels (Amersham Pharmacia) were used and were run in a Multiphore II apparatus with a constant current of 5mA per gel for two hours and 18mA per gel for 19 hours. Gels were fixed for 30 minutes with 10% methanol/7% acetic acid then stained for 16 hours in Sypro Ruby solution (Bio-Rad), destained for one hour with 10% methanol/7% acetic acid and scanned on the Bio-Rad Personal FX scanner. Protein profiling was repeated at least two times with cell wall proteins from each fungus. Gel images were analysed using the Melanie III image analysis program (Bio-Rad).

7.4.1.4 MALDI-Tof Mass spectroscopy analysis

SYPRO Ruby stained protein spots were excised from gels and subjected to a 16-hour tryptic digest at 37°C. The resulting peptides were extracted from the gels by sonication in 50% (v/v) acetonitrile, 1% (v/v) trifluoroacetic acid (TFA) for 10 minutes. Aliquots of the protein digests were spotted onto a sample plate containing α-cyano-4-hydroxycinnamic acid matrix and allowed to air dry. Matrix assisted laser desorption ionisation (MALDI) mass spectrometry was performed with a Micromass TofSpec IIE Time of Flight Mass Spectrometer (Manchester, UK). A nitrogen laser (337 nm) was used to irradiate the sample. The spectra were acquired in reflection mode in the mass range 600 to 3500 Da.

7.4.1.5 Analysis of peptide mass fingerprints

Peptide masses were assigned and used to search against SWISS-PROT and the translation of the EMBL DNA database called TrEMBL. These databases are available at http://expasy.proteome.org.au, an in-house mirror of the Swiss ExPASy site. The parameters used in the search were as follows: restrictions were placed on the species of origin (only fungi), the isoelectric points between 4 and 7 were selected, no restriction on molecular weight. For the protein spots to be unambiguously identified at least five peptides had to match.

7.5 Results and Discussion

Cell walls were isolated from *L. procerum*, *O. querci*, *O. ips*, *S. sapinea*, *Alternaria alternata*, *Trichoderma koningi* and *Epicoccum nigrum* grown in stationary culture to promote the production of high amounts of mycelia.

Among several methods tried for the fragmentation of mycelia, including bead beating, sonication, French Press, grinding in the presence of liquid nitrogen and bio-nebulising, it was found that a mixture of high pressure compression in the French Press and sonication were the most satisfactory methods in terms of quantity of material that could be processed and the size of mycelial fragments. The fragmented mycelia were further purified by differential centrifugation on a sorbitol cushion, which was reported to result in relatively pure mycelial material (Lim *et al*, 2001). When inspected under the light microscope, most of the mycelium was fragmented. This observation and the extensive washing of the fragmented mycelia indicated that the cytoplasmic material was successfully removed and that cell wall material was the only component left in the sample. Reproducible separation of cell wall proteins from all fungi tested was achieved by the extraction method described.

Preliminary experiments with pH gradients ranging from 3 to 10 showed the isoelectric points of the cell proteins from sapstain fungi and wood inhabiting fungi ranged between pH 4 and 7. The pH gradient 4 to 7 was therefore selected for further analysis.

Two-dimensional electrophoretic patterns of the cell wall proteins of sapstain fungi *O. ips*, *O. querci*, *L. procerum* and *S. sapinea* are given in Figure 7.1, Figure 7.2, Figure 7.3 and Figure 7.4 respectively. The two-dimensional electrophoretic patterns of the cell wall proteins of the wood inhabiting fungi *T. koningi*, *E. nigrum* and *A. alternata* are given in Figure 7.5, Figure 7.6, and Figure 7.7 respectively. Protein spots of interest (arrowheads in the specific figures) represented the unique protein spots that were further analysed.

About 220 protein spots were separated from the cell wall of *O. ips*, with the protein spots spread over an isoelectric point range from pH 4 to 7 (Figure 7.1). These protein spots included the weak, diffuse and sharp spots. At pH 4 to 7, most protein spots exhibited molecular mass smaller than 50kDa and many discrete spots smaller than 20kDa were detected. Thirty protein spots were excised from the gel in Figure 7.1, which were detected reproducibly and were dark enough to indicate sufficient protein content and were subsequently subjected to mass spectroscopy analysis.

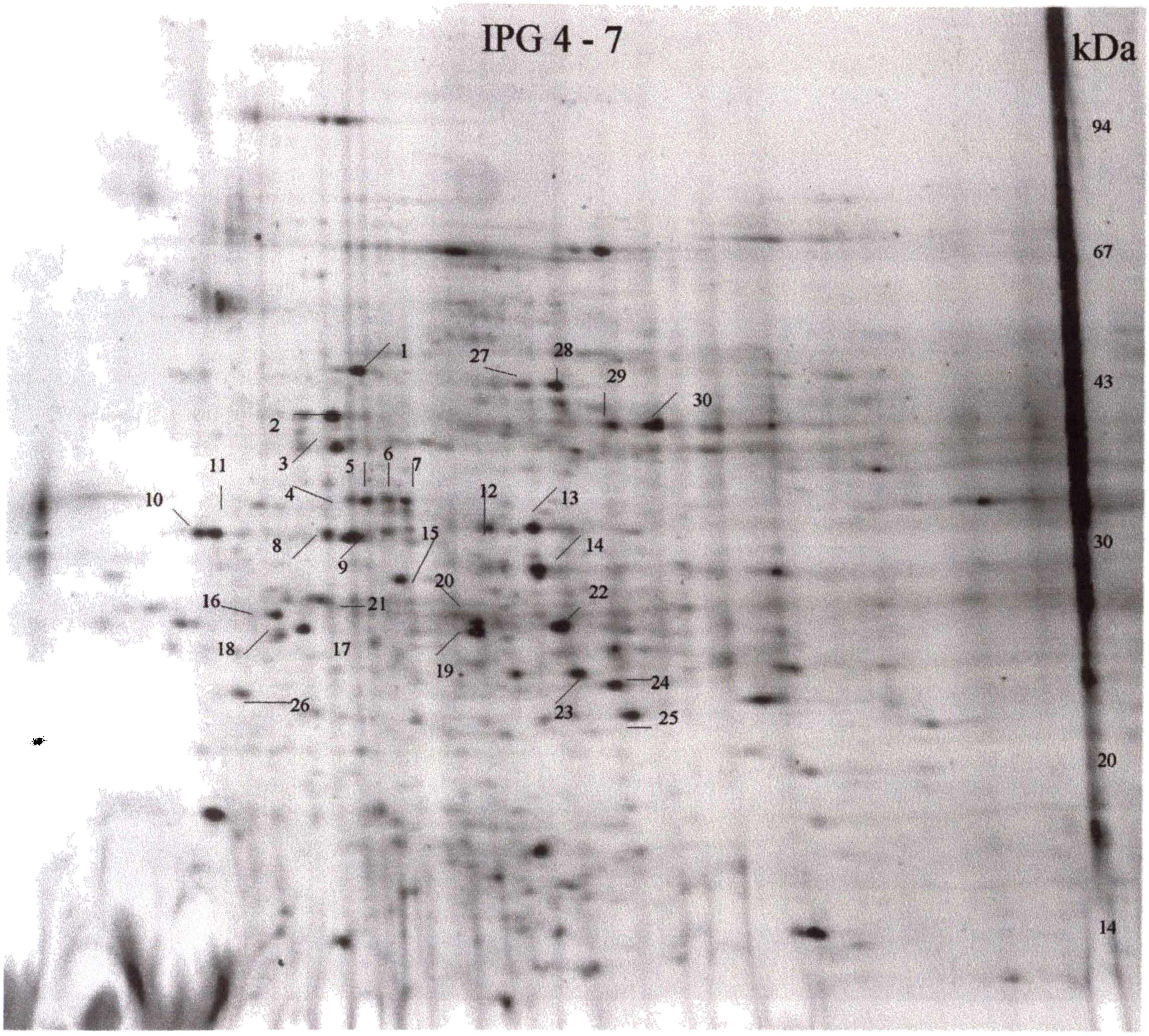


Figure 7.1: Protein pattern of the cell wall isolated from the sapstain fungus *O. ips*. Cell wall associated proteins were extracted and focused on a 17cm pH 4-7 gel. Second dimension separation was performed on 20cm 8-18% gradient gel. Arrow and numbers on the profile indicate spots that were excised and analysed by mass spectroscopy.

About 172 protein spots were separated from the cell wall of *O. querci*, with the protein spots spread over an isoelectric point range from pH 4 to 7 (Figure 7.2). At pH 4 to 7, most protein spots exhibited molecular mass smaller than 50kDa and many discrete spots smaller that 20kDa were detected. Twenty-three protein spots were excised from the gel in Figure 7.2, and were subsequently subjected to mass spectroscopy analysis.

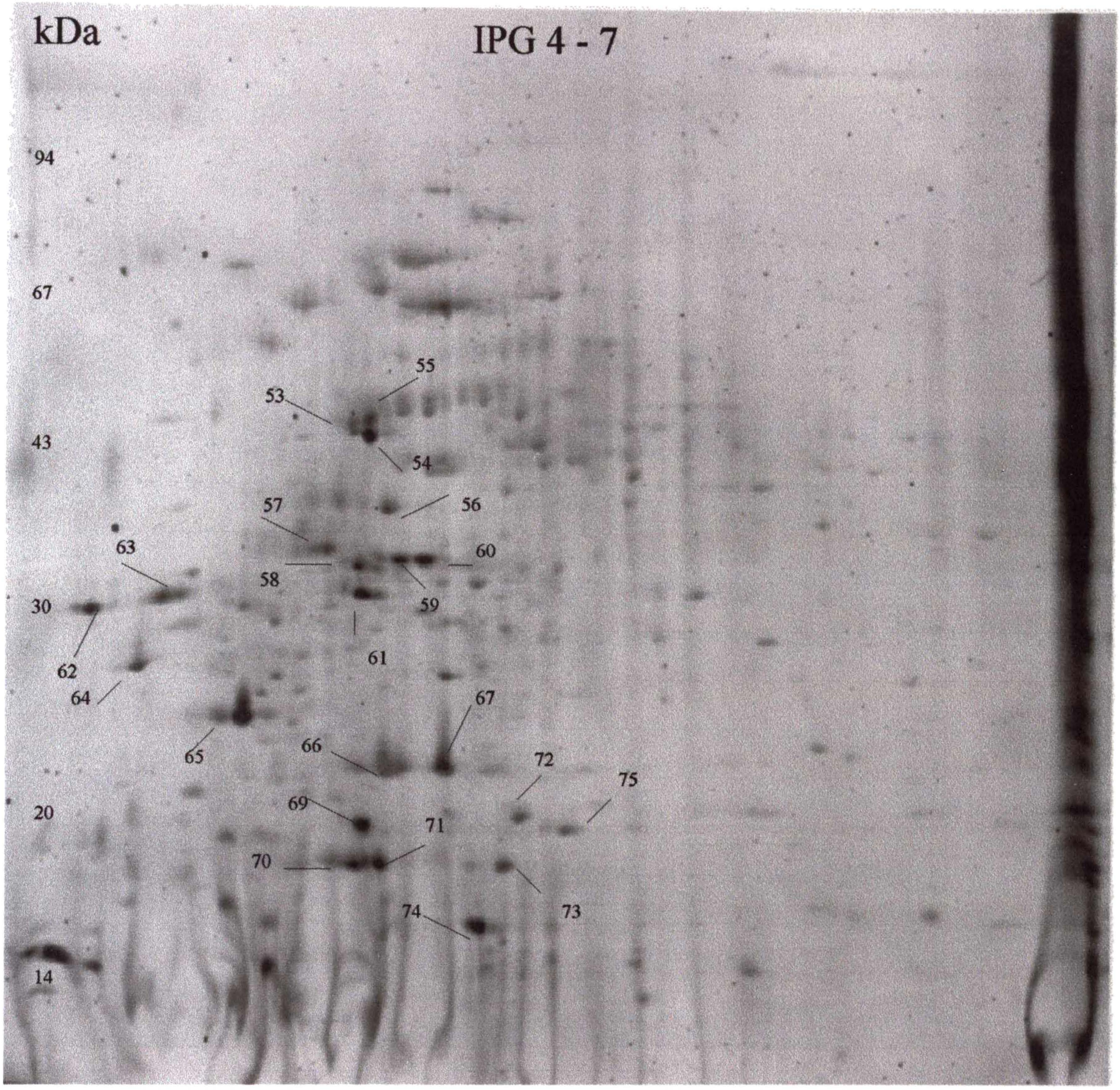


Figure 7.2: Protein pattern of the cell wall isolated from the sapstain fungus *O. querci*. Cell wall associated proteins were extracted and focused on a 17cm pH 4-7 gel. Second dimension separation was performed on 20cm 8-18% gradient gel. Arrow and numbers on the profile indicate spots that were excised and analysed by mass spectroscopy.

About 19 protein spots were separated from the cell wall of *L. procerum*, with the protein spots spread over an isoelectric point range from pH 4 to 7 (Figure 7.3). Most of the protein spots exhibited molecular masses that were smaller than 50kDa and were detected at pH 4 to 7. Eight protein spots were excised from the gel in Figure 7.3, and were subjected to mass spectroscopy analysis.

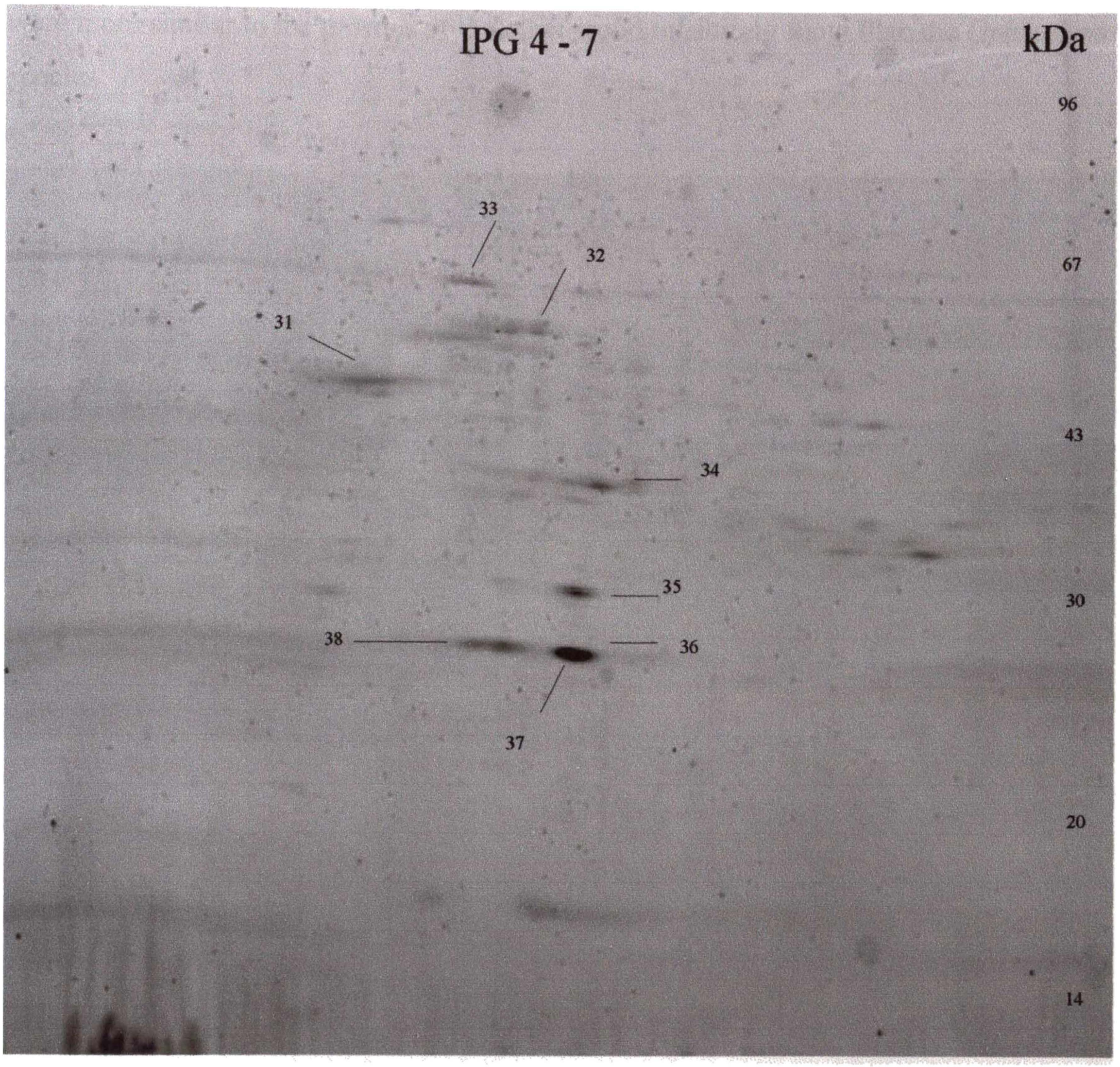


Figure 7.3: Protein pattern of the cell wall isolated from the sapstain fungus *L. procerum*. Cell wall associated proteins were extracted and focused on a 17cm pH 4-7 gel. Second dimension separation was performed on 20cm 8-18% gradient gel. Arrow and numbers on the profile indicate spots that were excised and analysed by mass spectroscopy.

About 33 protein spots were separated from the cell wall of the sapstain fungus *S. sapinea*, with the protein spots spread over an isoelectric point range pH 4 to 7 (Figure 7.4). Most of the protein spots exhibited molecular masses higher than 40kDa and were detected at pH 4 to 6. Nine protein spots were excised from the gel in Figure 7.4, and were subjected to mass spectroscopy analysis. Although *S. sapinea* is a sapstain fungus from the analysis of the proteome of this fungus it was evident that the cell wall proteins were more similar to the profiles of the other wood inhabiting fungi than the *Ophiostoma* species.

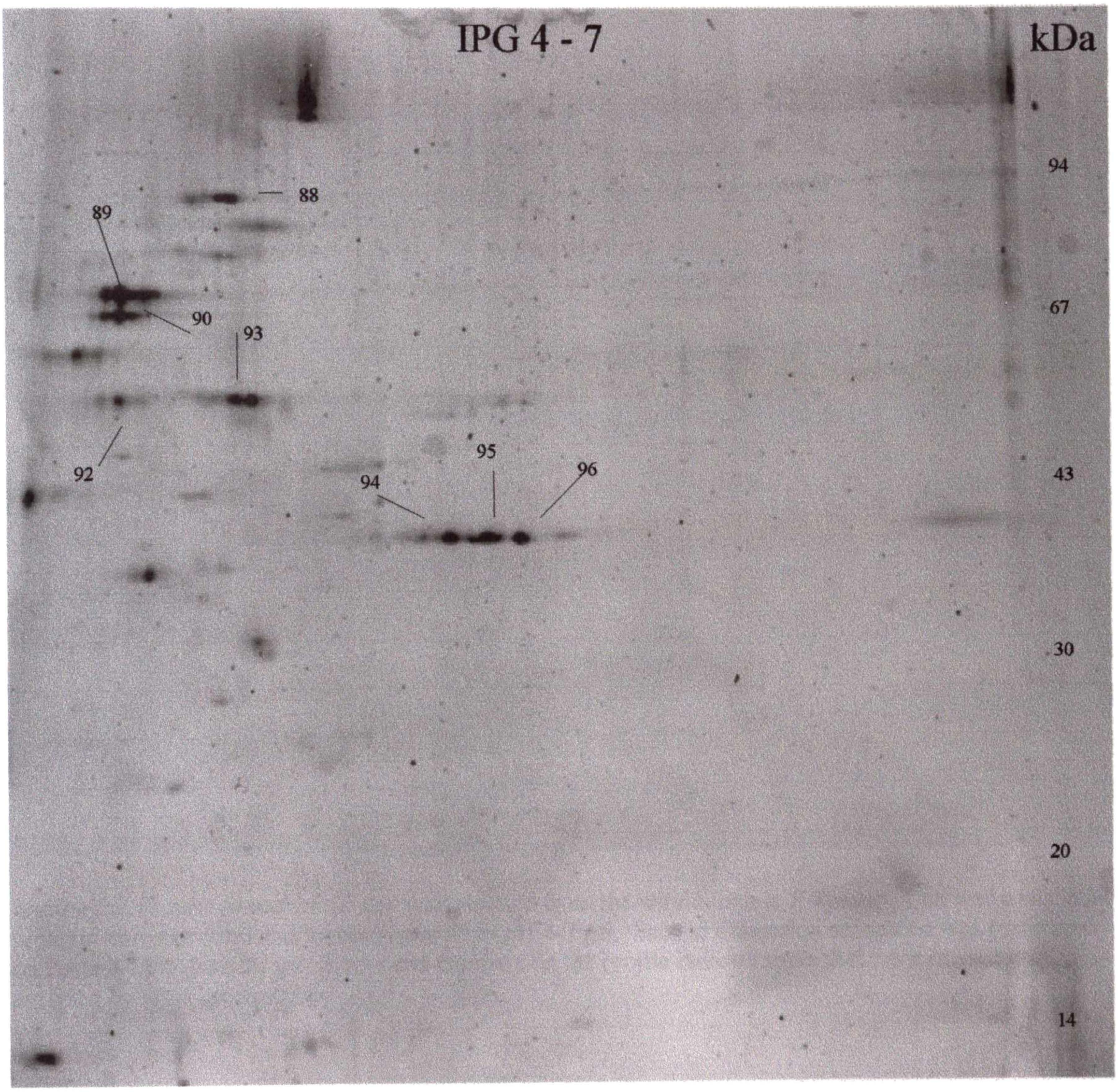


Figure 7.4: Protein pattern of the cell wall isolated from the sapstain fungus *S. sapinea*. Cell wall associated proteins were extracted and focused on a 17cm pH4-7 gel. Second dimension separation was performed on 20cm 8-18% gradient gel. Arrow and numbers on the profile indicate spots that were excised and analysed by Mass spectroscopy.

About 58 protein spots were separated from the cell wall of the mould fungus *T. koningi*, with the protein spots spread over an isoelectric point range from pH 4 to 7 (Figure 7.5). Most of the protein spots exhibited molecular masses greater than 30kDa. Twelve protein spots were excised from the gel in Figure 7.5, and were subjected to mass spectroscopy analysis.

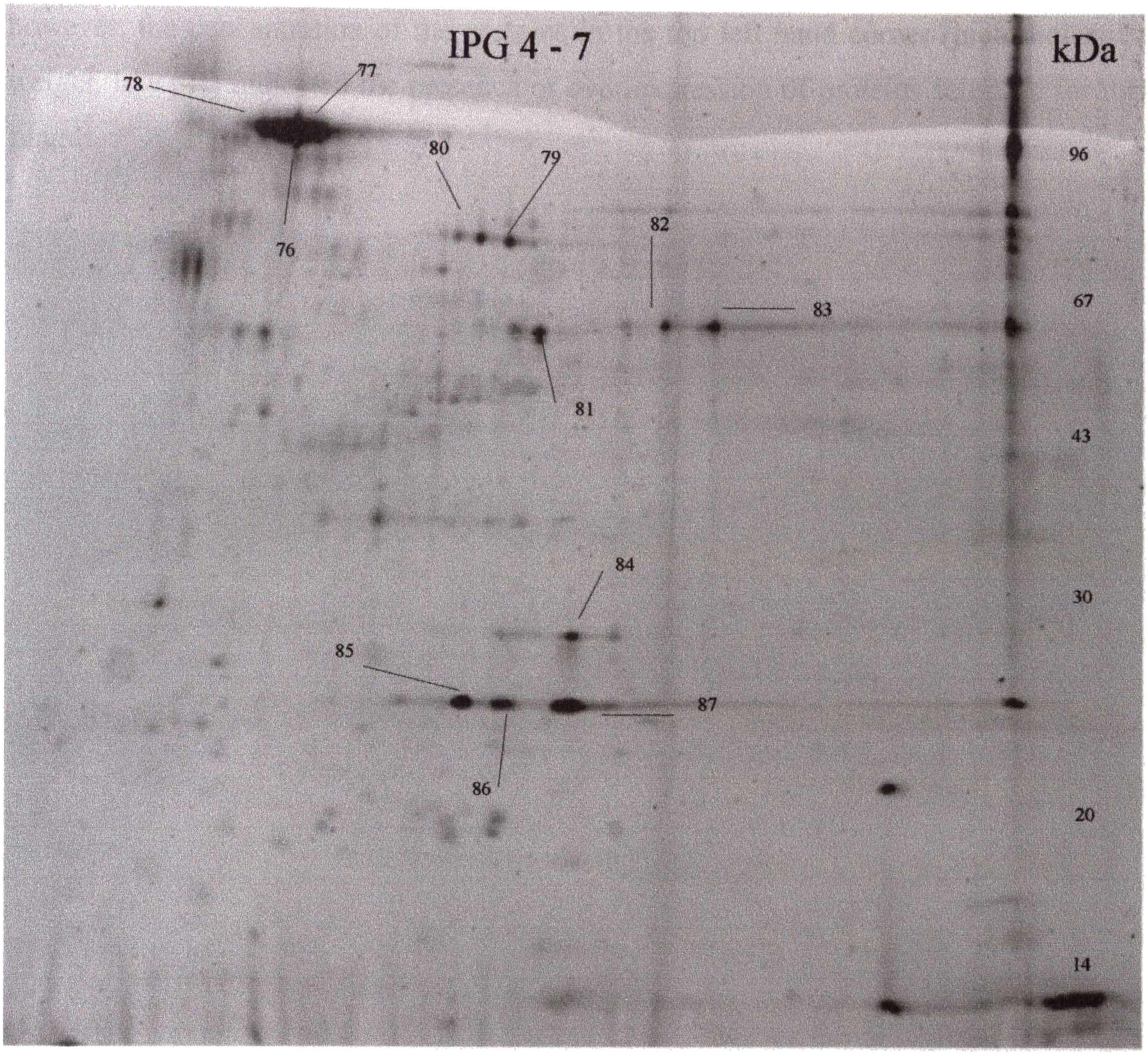


Figure 7.5: Protein pattern of the cell wall isolated from the mould fungus *T. koningi*. Cell wall associated proteins were extracted and focused on a 17cm pH 4-7 gel. Second dimension separation was performed on 20cm 8-18% gradient gel. Arrow and numbers on the profile indicate spots that were excised and analysed by mass spectroscopy.

The profile of the cell wall extracted proteins for the mould fungus *E. nigrum* is shown in Figure 7.6. There were approximately 35 protein spots separated by two-dimensional electrophoresis over an isoelectric point range of pH 4 to 7. Most of the proteins had high molecular weight and were in the range of pH 4 to 5. Four proteins were excised from the gel in Figure 7.6 and subjected to MALDI-Tof mass spectroscopy. *E. nigrum* had relatively less total protein content on the gel in comparison to *O. ips* (Figure 7.1), however, the concentration of the proteins in the top left hand corner (high molecular weight, low pH) indicated the presence or over-expression of proteins selective for this fungus.

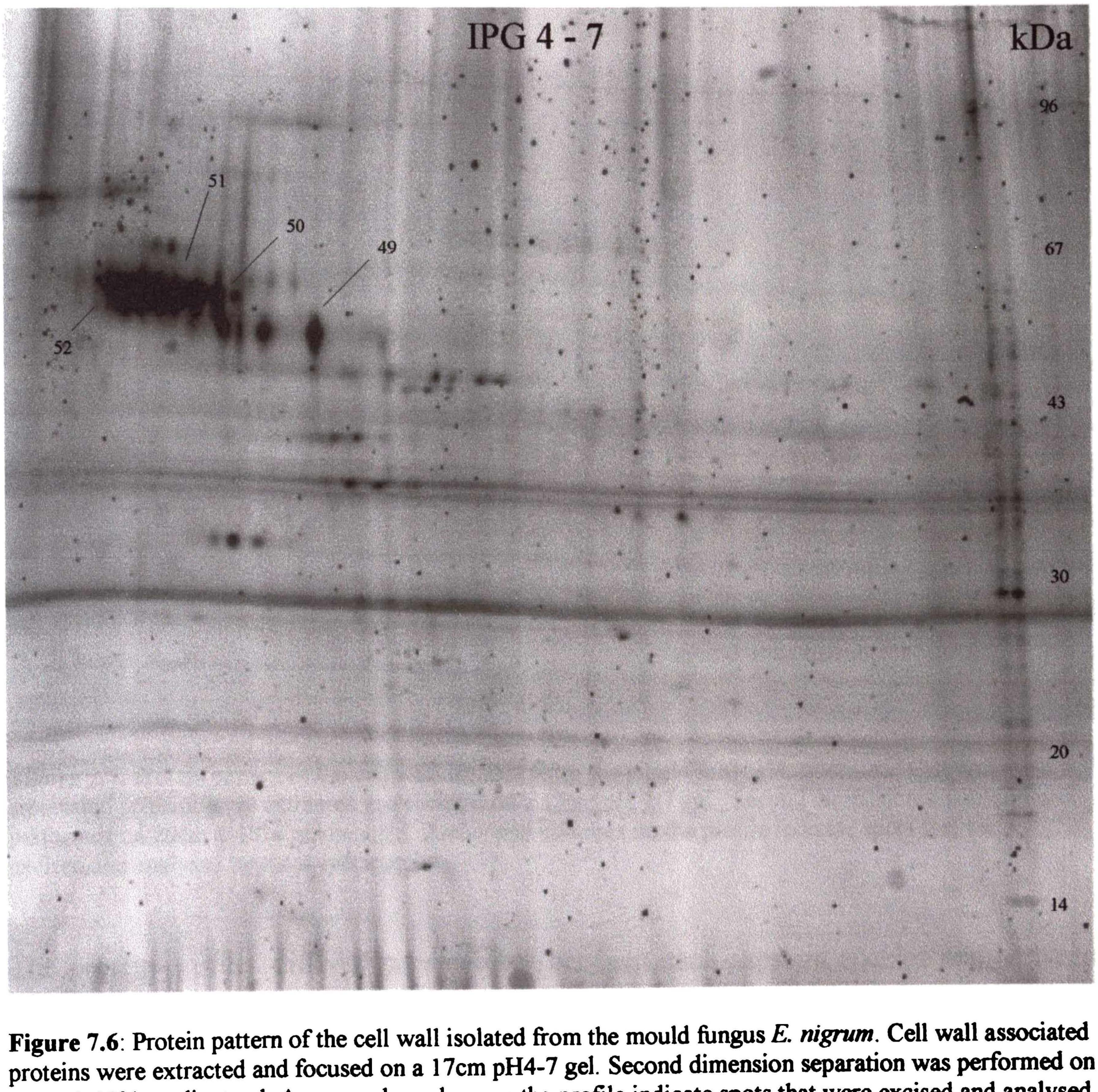


Figure 7.6: Protein pattern of the cell wall isolated from the mould fungus *E. nigrum*. Cell wall associated proteins were extracted and focused on a 17cm pH4-7 gel. Second dimension separation was performed on 20cm 8-18% gradient gel. Arrow and numbers on the profile indicate spots that were excised and analysed by mass spectroscopy.

About 51 protein spots were separated from the cell wall of *A. alternata*, with the protein spots spread over an isoelectric point range from pH 4 to 6 (Figure 7.7). Most protein spots exhibited high molecular masses greater than 60kDa. Ten protein spots were excised from the gel in Figure 7.7, and were subjected to mass spectroscopy analysis.

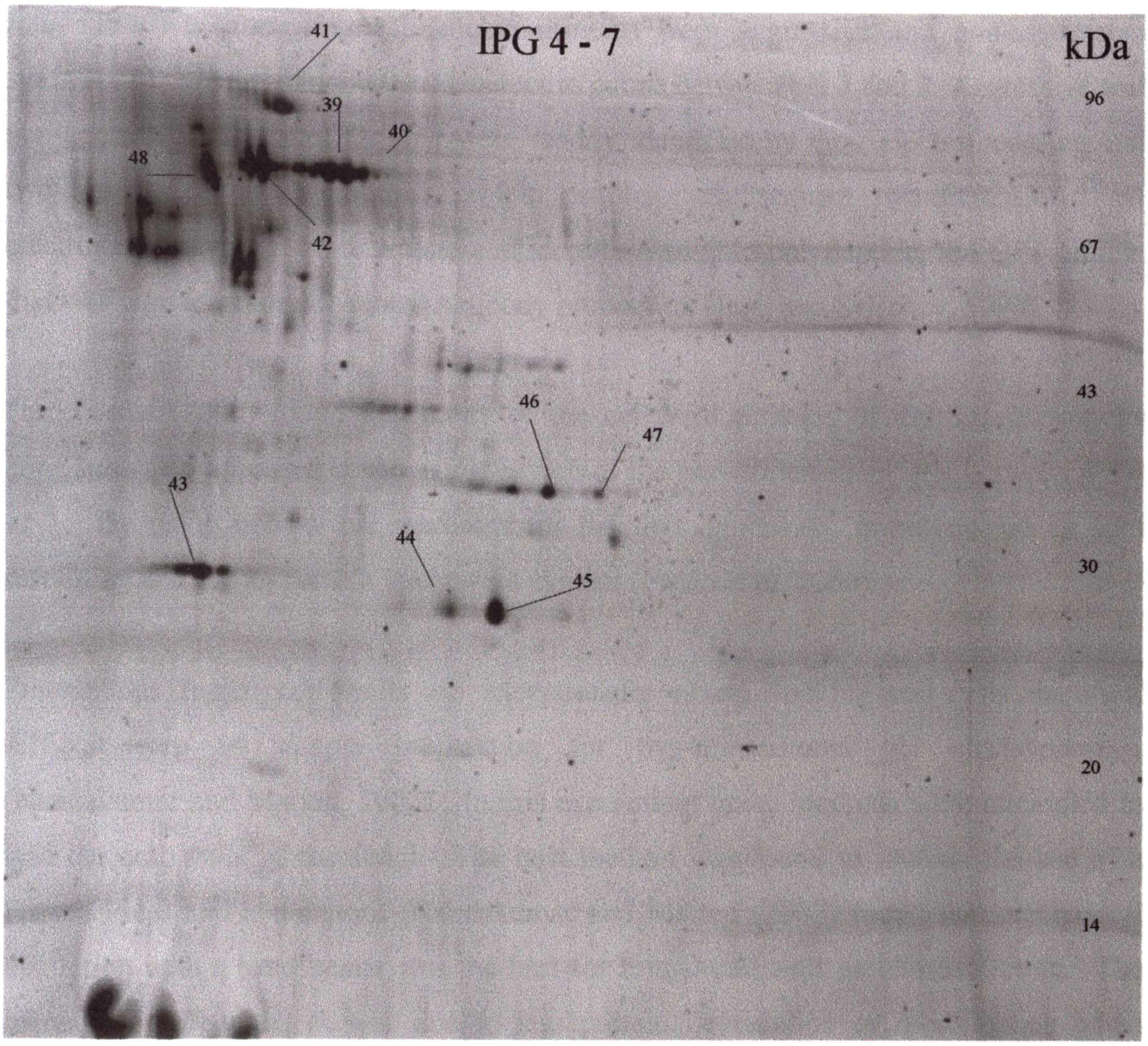


Figure 7.7: Protein pattern of the cell wall isolated from the mould fungus *A. alternata*. Cell wall associated proteins were extracted and focused on a 17cm pH 4-7 gel. Second dimension separation was performed on 20cm 8-18% gradient gel. Arrow and numbers on the profile indicate spots that were excised and analysed by mass spectroscopy.

The protein profiles differed considerably between each sapstain species and between the profiles of the other wood inhabiting fungi. Duplication of profiles from each species showed good repeatability indicating that cell lysis, protein solubilisation and two-dimensional electrophoresis protocols were reproducible for each species. The protein profiles of the sapstain fungi *L. procerum*, *O. ips*, and *O. floccosum* indicated a general distribution of proteins across isoelectric points ranging between pH 4 and 7 and molecular weight 10kDa and 150kDa. The protein profiles of the other wood inhabiting fungi (*E. nigrum*, *T. konigii*, and *A. alternata*) as well as the sapstain fungi *S. sapinea* in general showed a different expression pattern. For this group of fungi, there were a number of proteins expressed at high levels that appeared to have different isoforms and which occurred in the range pH 4 to 5 and molecular weight 60kDa to 120kDa.

Jeng (1986) compared fungal soluble proteins from aggressive and non-aggressive strains of *O. ulmi* and found their isoelectric points between pH 3 and 7. Aggressive and non-aggressive strains of *O. ulmi* were readily identified by their electrofocussing and two-dimensional electrophoresis protein profiles. Subsequent two-dimensional gel electrophoresis was used to isolate and identify a soluble polypeptide, the QP1 protein that was then used for polyclonal antibody production (Jeng and Svirchev, 1990).

Pardo *et al* (2000) found that many of the cell wall proteins of the *Saccharomyces cerevisiae* also appeared in the acidic region of the two-dimensional gels between pH 4 to 7. By using narrow pH gradients for the first dimension, improvements in spot resolution and higher protein loads were evident (Pardo *et al*, 2000).

Filamentous fungal cell walls are exceptionally strong, making cell lysis the most difficult step in sample preparation for two-dimensional gel electrophoresis (Nandakumar and Marten, 2002). In this experiment many methods were attempted to lyse the cell walls of the fungi. The best method was found to include the use of a French Press and sonication. Nandakumar and Marten (2002) found that mechanical disruption with a bead beater was the best for fungal cell wall protein extraction. The increase in effectiveness was due to less protein degradation or modification when compared to other methods. The methods used in this chapter were adequate to lyse the cell wall but in the future other methods maybe more successful.

A high degree of streaking and tailing was evident in all the gels. One of the major reasons for streaky two-dimensional gels and background smears is poor protein solubilisation (Görg *et al*, 2000). Special attention must be paid to cell lysis conditions, inactivation of protease activities, choice of adequate detergents, and the amount of reducing agents (Görg *et al*, 2000). The use of tributyl phosphine as a reducing agent reduces the streaking and gives excellent spot resolution (Walsh *et al*, 1998). Vertical streaks in the two-dimensional gels can be caused by salt fronts, protein aggregations or incomplete focusing in the first dimension (Görg *et al*, 2000).

There were only a few protein spots in the gel for the fungus *L. procerum*. Few protein spots are normally the result of inefficient extraction of proteins (Nandakumar and Marten, 2002).

Following two-dimensional electrophoresis of cell wall proteins of the sapstain species and wood inhabiting fungi, 96 proteins were selected for further analysis using MALDI-Tof mass spectroscopy. The monoisotopic peak lists of peptides from samples are provided in Appendix 2. Table 7.2 shows the representation of the fungi of interest within the SWISS-PROT and TrEMBL protein databases.

Table 7.2: Species representation in SWISS-PROT and TrEMBL protein databases.

Species	**Number of entries**
Total (All species, SWISS-PROT and TrEMBL)	435953
Ophiostoma species	29
O. ips	0
O. querci	0
Leptographium species	0
Sphaeropsis species	0
Trichoderma species	127
Trichoderma harzianum	37
Cladosporium species	30
Epicoccum species	0
Alternaria species	45
Alternaria alternata	32

At first, database searching using SWISS-PROT and TrEMBL protein databases was carried out against all fungi setting a minimum of five matching peptides to all unambiguous identification. Table 7.3 lists the score (% of matches with database proteins), the number of peptide matches, description of the theoretical protein matches with isoelectric points and molecular weights. Low confidence identifications were obtained for a few spots (Table 7.3). Searches were then carried out against all eukaryote entries. No high confidence identification could be made for any of the protein spots. This meant that none of the proteins corresponding to the 2-D gel spots were present in a protein database.

Table 7.3: Results from SWISS-PROT and TrEMBL searches comparing peptide mass of spots isolated from gels with theoretical proteins in the databases.

Spot Id	Spot number	Score	Number of Peptide matches	Acc. no. (SWISS-PROT)	Description	pI	Mw
A2	2	0.16	5	Q01886	HC toxin synthestase - *Cochliobolus carbonum*	5.8	574652
		0.16	5	Q12019	Midasin -*Saccharomyces cerevisiae*	4.9	559308
		0.16	5	Q94188	Phosphatidylinositol 3-kinase - *Crytococcus neofomans*	5.91	267218
		0.16	5	Q10105	Putative translational activator - *Schizosaccharomyces pombe*	5.6	297338
A3	3	0.32	9	Q09164	Cyclosporin synthetase - *Tolypocladium inflatum*	5.4	1689067
		0.21	6	Q94192	Chitin synthase - *Paracoccidiodes brasiliensis*	5.62	197292
		0.21	6	Q06554	RAD16 -*Saccharomyces cerevisiae*	6.1	180344
		0.21	6	Q8X1E9	Calcium channel - *Aspergillus nidulans*	7.59	237725
		0.18	5	P78716	Dynein heavy chain, cytosolic - *Fusarium solani*	5.66	493463
		0.18	5	P45443	Dynein heavy chain, cytosolic - *Neurospora crassa*	5.69	495578
		0.18	5	Q12019	Midasin - *Saccharomyces cerevisiae*	4.9	559308
		0.18	5	094116	Peptide synthetase - *Aureobasidium pullans*	5.27	543154
		0.18	5	Q92237	Nitrate reductase - *Fusarium moniliforme*	6.2	101817
		0.18	5	P78621	Cytokinesis protein sep A - *Aspergillus nidulans*	5.87	197356
		0.18	5	P40457	Hypothetical 195.1 kDa protein in DNA43-UBI1 intergenic region	5.8	195141
A6	6	0.26	7	Q09164	Cyclosporin synthetase - *Tolypocladium inflatum*	5.4	1689067
A7	7	0.3	6	Q09164	Cyclosporin synthetase - *Tolypocladium inflatum*	5.4	1689067
A9	9	0.28	5	Q09164	Cyclosporin synthetase - *Tolypocladium inflatum*	5.4	1689067
B2	14	0.32	7	Q09164	Cyclosporin synthetase - *Tolypocladium inflatum*	5.4	1689067
		0.23	5	P35207	Antiviral protein SKI2 - *Saccharomyces cerevisiae*	6.31	146059
B3	15	0.3	7	Q09164	Cyclosporin synthetase - *Tolypocladium inflatum*	5.4	1689067
B7	19	0.39	9	Q09164	Cyclosporin synthetase - *Tolypocladium inflatum*	5.4	1689067
		0.22	5	Q00083	Anucleate primary sterigmata protein - *Aspergillus nidulans*	5.09	183671
		0.22	5	P49376	ATP synthase beta chain, mitochondrial precursor - *Kluyveromyces lactis*	4.91	50519
		0.22	5	P23704	ATP synthase beta chain, mitochondrial precursor-*Neurospora crassa*	4.83	51931
C6	30	0.17	5	P29539	RIF1 protein - *Saccharomyces cerevisiae*	6.13	217961
C7	31	0.2	5	P87154	DNA polymerase epsilon - *Schizosaccharomyces pombe*	6.74	252887
		0.2	5	P45443	Dynein heavy chain - *Neurospora crassa*	5.69	495578
		0.2	5	Q09164	Cyclosporin synthetase - *Tolypocladium inflatum*	5.4	1689067
D1	37	0.22	5	Q09164	Cyclosporin synthetase - *Tolypocladium inflatum*	5.4	1689067
D4	40	0.5	6	Q09164	Cyclosporin synthetase - *Tolypocladium inflatum*	5.4	1689067
D5	41	0.3	8	Q09164	Cyclosporin synthetase - *Tolypocladium inflatum*	5.4	1689067
		0.19	5	P45443	Dynein heavy chain - *Neurospora crassa*	5.69	495578
D6	42	0.26	6	Q09164	Cyclosporin synthetase - *Tolypocladium inflatum*	5.4	1689067
		0.22	5	060072	Pulative helicase - *Schizosaccharomyces pombe*	5.81	218565
D7	43	0.23	5	Q9P7T1	Pulative peptide synthetase - *Schizosaccharomyces pombe*	5.9	559849
E5	53	0.3	6	P23704	ATP synthase beta chain, mitochondrial precursor - *Neurospora crassa*	4.83	51931
E7	55	0.27	7	Q09164	Cyclosporin synthetase - *Tolypocladium inflatum*	5.4	1689067
		0.19	5	074126	MUS38 - *Neurospora crassa*	5.73	100375
		0.19	5	Q9P7T1	Putative peptide synthetase - *Schizosaccharomyces pombe*	5.9	559849
		0.19	5	Q07878	Vacuolar protein sorting - associated protein VPS13 - *Saccharomyces cerevisiae*	5.41	357849
E12	60	0.17	6	094248	Hypothetical coiled coil protein - *Schizosaccharomyces pombe*	5.06	537787

Table 7.3: continued

Spot Id	Spot number	Score	Number of Peptide matches	Acc. no. (SWISS-PROT)	Description	*pI*	Mw
F1	61	0.3	7	Q09164	Cyclosporin synthetase - *Tolypocladium inflatum*	5.4	1689067
		0.26	6	P08964	Myosin-1 isoform - *Saccharomyces cerevisiae*	6.06	223636
		0.22	5	Q990022	14-3-3 protein homolog - *Trichoderma harzianum*	5.8	29998
		0.22	5	Q01886	HC toxin synthestase - *Cochliobolus carbonum*	5.8	574652
		0.22	5	042926	Putative vacuolar protein - *Schizosaccharomyces pombe*	5.2	354025
		0.22	5	Q12721	Acetyl CoA carboxylase - *Ustilago maydis*	5.89	240031
		0.22	5	Q9HEN0	14-3-3-like protein - *Trichoderma reesei*	4.79	29597
		0.22	5	Q9P4Z1	Related to TOM1 protein - *Neurospora crassa*	4.96	452578
		0.22	5	P50530	Serine/threonine-protein kinase - *Schizosaccharomyces pombe*	6.14	78595
F7	67	0.25	6	Q09164	Cyclosporin synthetase - *Tolypocladium inflatum*	5.4	1689067
G2	74	0.21	6	Q8X0C5	Related to kinesin-like protein - *Neurospora crassa*	5.94	216644
		0.18	5	Q9P4Z1	Related to TOM1 protein - *Neurospora crassa*	4.96	452578
G5	77	0.33	7	Q09164	Cyclosporin synthetase - *Tolypocladium inflatum*	5.4	1689067
		0.29	6	Q9P7T1	Putative peptide synthetase - *Schizosaccharomyces pombe*	5.9	559849
		0.24	5	020981	DNA polymerase - *Cryphonectria parasitica*	6.79	138858
		0.24	5	094125	DNA polymerase - *Cryphonectria parasitica*	6.64	138957
		0.24	5	P07259	URA1 protein - *Saccharomyces cerevisiae*	5.59	245126
G6	78	0.3	7	Q09164	Cyclosporin synthetase - *Tolypocladium inflatum*	5.4	1689067
		0.26	6	Q9P7T1	Putative peptide synthetase - *Schizosaccharomyces pombe*	5.9	559849
		0.22	5	020981	DNA polymerase - *Cryphonectria parasitica*	6.79	138858
		0.22	5	094125	DNA polymerase - *Cryphonectria parasitica*	6.64	138957
		0.22	5	P07259	URA1 protein - *Saccharomyces cerevisiae*	5.59	245126
G7	79	0.29	5	Q09164	Cyclosporin synthetase - *Tolypocladium inflatum*	5.4	1689067
H1	85	0.28	7	Q09164	Cyclosporin synthetase - *Tolypocladium inflatum*	5.4	1689067
		0.2	5	Q9P7T1	Putative peptide synthetase - *Schizosaccharomyces pombe*	5.9	559849
H2	86	0.21	5	Q09164	Cyclosporin synthetase - *Tolypocladium inflatum*	5.4	1689067
H3	87	0.26	6	Q09164	Cyclosporin synthetase - *Tolypocladium inflatum*	5.4	1689067
H4	88	0.46	6	Q09164	Cyclosporin synthetase - *Tolypocladium inflatum*	5.4	1689067
H9	93	0.38	5	P45443	Dynein heavy chain - *Neurospora crassa*	5.69	495578

No proteins listed in Table 7.3 are commonly identified in the cell wall of *S. cerevisiae*. Pardo *et al* (2000) identified a number of cell wall proteins from *S. cerevisiae* using a proteomic approach. The most commonly isolated proteins included Glycosylphosphatidylinositol (GPI) proteins, Pir proteins or cell wall hydrolytic enzymes.

Peptide mass fingerprinting is complicated in a number of ways and can lead to ambiguous database search results. For example, it is not usual to recover only a few tryptic peptides from a digest, particularly when the digest is carried out on low level proteins in the gel (Keough *et al*, 2000). This limits the protein sequence coverage obtained from the experimental tryptic mass map and often limits the specificity of corresponding database searches (Keough *et al*, 2000). Comigrating proteins produce mixtures of tryptic peptides that may confound database searches. Chemical modification of peptide sequences (phosphorylation, glycosylation or oxidation) produces tryptic masses that differ from the theoretical values contained in the databases (Keough *et al*, 2000). These complications led to the development of a number of approaches to improve database search specificity beyond that obtainable with simple tryptic mass fingerprinting. They include the use of appropriate search filters (protein molecular weight, isoelectric point or species origin) or the use of partial peptide sequences obtained by tandem mass spectrometry (Keough *et al*, 2000).

As shown in this chapter the data collected by MALDI mass fingerprinting was not sufficient for reliable identification of the protein spots. MALDI identification by peptide mass fingerprinting requires that the full-length gene be present in the databases. The success rate of this method will receive an additional boost with the availability of all predicted genes in sequence databases (Pandey and Mann, 2000). In order to successfully identify the protein spots obtained by two-dimensional gel electrophoresis other methods may prove better than MALDI analysis. For this thesis, these methods were not employed due to budget restraints on the project.

To generate additional data, several other paths could be used: one could simply remeasure the spectrum with different parameters to improve the spectrum quality; one could generate an additional fingerprint with a protease of different specificity; or one could use peptide fragmentation techniques such as tandem mass spectroscopy (Lahm and Langen, 2000).

Amino acid fragmentation data generated by Tandem mass spectroscopy (MS/MS) is specific to an individual peptide. When numerous peptide ions enter an MS/MS, one peptide mass to charge ratio value can be isolated and then dissociated to obtain a fragmentation pattern that is indicative of the amino acid sequence. The ability to search a database of sequences to match a tandem mass spectrum uniquely to a sequence allows proteins in mixtures to be identified (Yates, 2000). Lim *et al* (2001) found that when peptide mass fingerprinting was applied to identify the spots isolated from *Trichoderma reesei* none were identified in the database. However with tandem mass spectrometry nine protein spots gave a peptide sequence (Lim *et al*, 2001).

Another approach to identify the protein spots obtained in the gels could be analysis of amino acid composition. This method provides a rapid and inexpensive means of screening large numbers of proteins for homologous entries within databases. However, the quality of amino acid analysis, the accuracy of the computer-derived score and inconclusive matches due to other proteins sharing a similar amino acid composition means that adequate results cannot always be obtained (Cordwell *et al*, 1995). The combined use of amino acid composition and MALDI-Tof mass spectroscopy could allow a high degree of confidence to be placed on identifications because they are based upon 20 homologous data sets of at least 20 parameters (16 amino acids and 4-10 tryptic digest fragments) (Cordwell *et al*, 1995).

Due to a limited budget for this project the other options were not investigated. In the future it may be possible to use these methods to obtain the desired unique protein, which could then be used in development of an antibody based identification system for sapstain fungi. The methods of identification investigated in this chapter look promising to aid in the identification of sapstain fungi in the future.

Chapter 8 – General Discussion and Conclusions

This research evolved from an original aim to study management and control of sapstain fungi in New Zealand particularly relevant to developing early detection methods. Early in the research it became clear that there were significant gaps in fundamental knowledge of the occurrence of sapstain fungi in New Zealand and the key parameters affecting their colonisation of *P. radiata.* The development of the proteomic analysis, which was undertaken as a collaboration of the thesis research with the Australian Proteomic Analysis Facility in Sydney, produced results not promising in obtaining unique protein sequences to further develop antibodies for detection. A decision was subsequently made to make the thesis more broadly defined and addresses ecology of sapstain fungi in New Zealand and relevant to commercial forestry.

The nationwide survey of New Zealand sapstain fungi identified the presence of *S. sapinea*, thirteen known *Ophiostoma* species and a number of undefined species of *Ophiostoma* (Farrell *et al*, 1997; Farrell *et al*, 1998; Kay *et al*, 1998). Information on this survey was presented as preliminary reports at symposia (Farrell *et al*, 1997; Farrell *et al*, 1998; Kay *et al*, 1998). These reports included lists of the sapstain species found at the time of publication (1997 and 1998) but no specific results and conclusions were ever developed from the raw data on the ecology and distribution of each species within New Zealand. The data collected in this survey was subsequently evaluated in this thesis research according to ecological criteria including geographical and temporal distribution and was presented in Chapter 3. This study gave a greater understanding of the ecology of sapstain fungi in New Zealand.

Re-evaluation of the survey data found seventeen individual species of *Ophiostoma* and *S. sapinea* in New Zealand. No sapstain fungi predominated in any geographical area of New Zealand. The diversity of sapstain fungi was greatest in areas with high proportions of plantation forests. More *Ophiostoma* species compared to *S. sapinea* were cultured from *P. radiata* plantations and processing sites however native forest areas and other areas including nurseries, urban areas and farm areas had relatively high proportions of *S. sapinea*. *S. sapinea* was more prominent in the general forest samplings (needles, cones and other forest debris) than the *Ophiostoma* species. From logs, timber, wood chips and plywood, more *Ophiostoma* species than *S. sapinea* were cultured. *S. sapinea* was found more in spring and summer, while *Ophiostoma* species were more abundant in the autumn and winter months. By investigating aspects of ecology of sapstain fungi in New Zealand this research has provided valuable information that may be incorporated into the management of sapstain. Two key conclusions are made from this work i) *S. sapinea* the most common sapstain fungi in New Zealand has the most impact when logs or lumber are left in the forest environments, and two ii) *Ophiostoma* species have greater profusion in processing areas and greater bearing on export logs and lumber than *S. sapinea.*

As there were a number of sapstain species found in the survey, the question was asked whether these fungi were the same species found in logs destined for export. Two trials were subsequently developed that followed the sapstain fungi from harvesting to the export destination, Japan. These trials were the first reports of stain development and fungal colonisation at harvesting, at the New Zealand port and at the export destination. The trials also measured the effect the time from harvesting to anti-sapstain treatment, the effect of season and the effect of crossing the equator on development of sapstain. From Export Trial I (New Zealand summer), nine species were isolated, the most common being *O. floccosum, O. querci* and *O. setosum.* Export Trial II (New Zealand winter) had a different combination of nine sapstain species detected, the most common were *S. sapinea, O. querci, O. floccosum, O. setosum* and *O. piceae.* Logs arriving in Japan that were harvested in New Zealand summer had more stained logs and more severity of stain than logs harvested in winter. Results from this trial showed that anti-sapstain treatment within one day was more effective at controlling sapstain than when logs were treated four days post harvesting. This research showed the New Zealand sapstain fungi being exported on logs are generally already present in Japan and no serious pathogens were detected.

A summary of the individual sapstain fungi isolated in the survey of New Zealand sapstain fungi (Chapter 3) and the two Export Field Trials (Chapter 4) is provided in Table 8.1. A comparison of the species isolated shows that in the majority of cases the most dominant fungi in the survey were also isolated in high numbers in the export trials with the exceptions of *O. ips* and *O. piliferum. O. ips* as discussed previously in Chapter 5 had a growth optimum between 25 and 30°C (Lindgren, 1942; Kay and Ah Chee, 1999). This fungus was isolated in all seasons in the survey (Chapter 3) but only from areas in the North Island and the top of the South Island. *O. ips* was not isolated in the export trial established in the winter or unable to successfully colonise in the winter colonisation trial presented in Chapter 5. It is suggested that the staining fungi *O. ips* is mainly a problem for the forest industry in New Zealand in summer.

O. piliferum was only isolated occasionally from the export trials, but was the third most common sapstain fungi in the survey. This fungus is also the most commonly found sapstain fungus in North American (Olchowecki and Reid, 1974; Seifert, 1993). *O. piliferum* had the fastest growth on malt extract agar between 20 and 25°C (Miller and Goodell, 1981; Kay and Ah Chee, 1999). It is unclear why few isolations of *O. piliferum* occurred in the export trials. It is interesting to note that in previous trials with the commercially available albino *O. piliferum* strain, Cartapip 97 by the candidate it was found that this fungus was unable to successfully colonise *P. radiata* logs (Thwaites, 1999).

Table 8.1: Summary of sapstain fungi isolated and identified in New Zealand survey and export trials.

	Frequency of isolations of each species		
Sapstain species	**Survey**	**Export trial 1**	**Export trial 2**
L. procerum	83	45	3
L. truncatum	3		
Ophiostoma sp. E	14		
O. floccosum	218	241	50
O. galeiformis	12		
O. huntii	33	52	4
O. ips	299	10	
O. nigrocarpum	1		
O. piceae	52	40	48
O. piliferum	146	1	3
O. pluriannulatum	10		1
O. querci	80	192	98
O. setosum	52	122	51
O. stenocerus	4		
Pesotum fragans	1		
S. sapinea	562	57	181
Sporothrix sp. D	2		

At the time the survey data was collected it was believed that only one member of the *Botryosphaeria* genus, *S. sapinea* was in New Zealand (Birch, 1936; Butcher, 1967; Chou, 1976a; Farrell *et al*, 1998). Recently, an unidentified *Botryosphaeria* species was found as an endophyte in *P. radiata* trees from Kinleith Forest (Harrington, personal communication). For the data collected in the survey and presented in Chapter 3, therefore, it is uncertain whether *Botryosphaeropsis* species was isolated and not correctly identified. *Botryosphaeria* species were not isolated in any of the following trials presented in this thesis. Two isolates of *Botryosphaeria* species were inoculated onto *P. radiata* specimens in Chapter 5 and this fungus causes an extensive dark grey to black stain. The impact of this sapstain fungus to the New Zealand forest industry is unknown and requires more investigation. Other *Botryosphaeria* species cause extensive problems to the forest industries in other countries. *Lasiodiplodia theobromae* (syn. *Botryodiplodia theombromae*) is one of the most frequently cited fungi as early coloniser of tropical timber (Eaton and Hale, 1993; Encinas and Daniel, 1995). The staining of wood due to *L. theobromae* was studied, particularly in relation to its prevention and control (Tabirih and Seehan, 1984; Encinas and Daniel, 1995).

High numbers of *Ophiostoma* species were isolated in both trials to Japan, indicating that these fungi should be of more concern than the sapstain fungus *S. sapinea* to the forest industry in New Zealand regarding export logs and potential biosecurity issues.

The interactions between *S. sapinea* and *Ophiostoma* species were studied in the ecological investigation of sapstain fungi in New Zealand (Chapter 3) and in the examination of logs from harvesting to export (Chapter 4). It was not possible to fully investigate the nature of this relationship, beyond documenting the dominance of *S. sapinea* and total *Ophiostoma* species according the particular criteria assessed. There was a seasonal preference of *S. sapinea* in spring and summer, while *Ophiostoma* species were dominant in the autumn and winter. *S. sapinea* was more prominent in the general forest samplings (needles, cones and other forest debris) than the *Ophiostoma* species which dominated in logs, timber, wood chips and plywood. *S. sapinea* and *Ophiostoma* species generally appear to have different niches. Whether the differences are due to the means of disseminating of each group of species or the seasonal effect was speculated.

Reay *et al* (2002) described the significant relationship between sub-lethal attack of seedlings by the introduced bark beetle *Hylastes ater* and the invasion by many sapstain fungi. The flight activity of *H. ater* is predominantly in the autumn months in New Zealand (Reay and Walsh, 2001). The sticky spores on the synnema and perithelial stalks of *Ophiostoma* species, are disseminated by wind and insect vectors, and are commonly found on logs and timber (Dowding, 1970). The insect vectors, particularly the bark beetles are found both in logs and stumps (Reay and Walsh, 2001). Insect and wind increases the spread of *Ophiostoma* species from logs processing sites and ports, therefore more inoculum potential for *Ophiostoma* species should occur at these sites. The relationship between the presence of the greater numbers of *Ophiostoma* species in autumn and the flight activity of *H. ater* maybe related and needs further investigation.

The spores of *S. sapinea* are associated with cones and needles (Palmer *et al*, 1988) that are found more commonly on the forest floor. *S. sapinea* sporulates prolifically on cones, after they have opened and discharged seeds (Peterson, 1977; Chou, 1984). The inoculum produced on these retained cones plays an important role in the survival of the fungus. The conidia produced on these cones may infect current year shoots and also the second year seed cones. The inoculum density of *S. sapinea* is higher therefore in the forest environment. Brookhouser and Peterson (1971) described the dissemination of *S. sapinea* spores as only occurring during periods of rainfall and at an optimum temperature of 24°C for germination. In this survey there were more isolations of *S. sapinea* in spring and summer but this fungus was also isolated in the winter and autumn. Swart and Wingfield (1991) found that spring was the most infectious time for *S. sapinea* into pruning wounds, relating to higher temperatures prevailing after periods of maximum rainfall.

Many investigations studied the impact of temperature on the growth of sapstain fungi (Lagerberg *et al*, 1927; Lindgren, 1942; Reynolds *et al*, 1972; Käärik, 1980; Miller and Goodell, 1981). The majority of this work found that individual species of *Ophiostoma* varied in their temperature optima and ranges.

The growth and development of sapstain fungi is strongly influenced by the nutrient, moisture and oxygen content of the wood as well as the ambient temperature (Seifert, 1993). This is exemplified in New Zealand by the variation in the incidence of sapstain development between winter and summer as seen in Chapter 4. A number of the *Ophiostoma* species collected during the survey of New Zealand sapstain fungi (Chapter 3) were encountered which were not well studied with regard to colonisation and stain formation on New Zealand *P. radiata*. This led to the establishment of a series of experiment in the laboratory to evaluate colonisation and stain development at various temperatures. Field trials were also created to estimate colonisation in two seasons, New Zealand summer and winter. Temperature affected the growth of various species of sapstain fungi on synthetic media and on *P. radiata*. *O. ips* and *S. sapinea* grew more rapidly on both synthetic media and *P. radiata* specimens at higher temperatures. *O. floccosum* and *L. procerum* grew more rapidly at 23°C. In the field environment *O. floccosum* was the most successful coloniser of *P. radiata* logs in both winter and summer. Stain developed on all treatments 15 days after inoculation in the summer field trial, however no stain developed on any treatment in the winter field trial. Environmental factors like temperature and moisture content affected stain development. A number of sapstain species were also evaluated for sapstain development on laboratory specimens of *P. radiata*. *Botryosphaeria* species produced the most extensive dark grey to black stain of all the sapstain species assessed. *O. huntii*, *O. galeiformis*, *L. truncatum* and *L. procerum* produced a light grey stain on *P. radiata*. *O. setosum*, *O. stenocerus* and *O. nigrocarpum* slightly discoloured the wood. *O. querci* and *O. coronata* produced no appreciable stain. The colonisation of *P. radiata* by sapstain fungi was analysed under the microscope. No differences were observed with regard to the spatial distribution of the sapstain fungi. Hyphae of sapstain fungi grew in parenchyma cells and tracheids. The hyphae of the sapstain fungi passed from one wood cell to another by growing through the pit membranes or by direct cell wall penetration using an appressorium. From these results a better understanding of the biology of sapstain species is emerging. Such knowledge on the mechanisms and environmental triggers of growth and stain development will aid in the more advanced technologies to successfully control sapstain.

An important finding in the colonisation experiments was the impact of environmental stresses on melanin production. Melanin was proposed to provide protection against various environmental threats including desiccation, ultra violet and visible light irradiation, fungicides and extremes of temperature (Bloomfield and Alexander 1967; Brasier 1978; Bell and Wheeler, 1986). There has been some research on the formation and biosynthesis of melanin in sapstain species. Zink *et al* (1989) showed that the hyphae of two sapstain fungi (*C. coerulescens* and *A. alternata*) had deposits of melanin in the cell walls in the form of globular granules (Zink *et al*, 1989). Zimmerman *et al* (1995) found that *O. piliferum* produced melanin via DHN in penetaketide biosynthesis. Research by Eagen *et al* (1996) found genes from *A. alternata's* pentaketide pathway. They used a heterologous probe to screen southern blots from other sapstain fungi and detected the presence of homologous copies of the *A. alternata* DHN genes in the sapstain fungi, *O. floccosum* (Eagan *et al*, 1996). Further investigations by Eagen *et al* (2001) and Wang *et al* (2001) isolated and characterised the OSD1, a gene encoding scytalone dehydratase from *O. floccosum*. The mechanism of the induction of biosynthesis of melanin in sapstain fungi and the factors that possibly contribute to melanin induction is not fully known (Kreber *et al*, 1999). More extensive research on albino strains of *Ophiostoma* that lack the melanin pigment may provide some answers to the mechanism of induction due to environmental stresses.

Newly developed albino strains of *O. floccosum*, *O. piceae* and *O. pluriannulatum* were investigated as biological control agents in the field as previous research on using albino technology proved successful on *Pinus resinosa* and southern yellow pine in North America (Blanchette *et al*, 1992; Farrell *et al*, 1993; Behrendt *et al*, 1995a, b). These studies demonstrated that applying an albino strain of *O. piliferum* marketed under the name Cartapip 97 to freshly cut logs allowed the inoculated strain to preferentially colonise the sapwood thereby capturing nutrient resources and inhibiting subsequent colonisation by organisms that would occupy the same ecological niche such as the dark staining fungi.

Albino isolates of *O. floccosum*, *O. piceae* and *O. pluriannulatum* are now developed. Albino isolates *O. pluriannulatum* were demonstrated to be effective by this thesis research and in collaboration with colleagues at the University of Minnesota in field trials in New Zealand on mature *P. radiata* logs to control sapstain (Held *et al*, in press). This study gave a greater understanding of the biology of albino strains of *Ophiostoma* and their growth in synthetic media and on *P. radiata* compared to wild type *Ophiostoma* species. The mode of action of the albino strains mainly through the use of primary resource capture. Albino *O. floccosum* strain were shown however to produce an antibiotic affect when challenged with *S. sapinea*.

This thesis provided results from four field trials showing variation in the biological control ability of the albino strains against wild type sapstain fungi. The first two trials showed significant differences among strains used for biological control of sapstain. Therefore, the inoculation of any albino fungal strain on freshly harvested logs was not sufficient to control sapstain. The data from Chapter 5 and 6 concluded that the ability of certain strains of wild type and albino isolates to grow and colonise wood were dependent on temperature and moisture content as well as the antagonistic response to other wood inhabiting fungi. These results demonstrate the need for more investigation into the biology of the albino strains in order to select for more effective control fungi.

To develop the albino technology further, the response of new strains to temperature, moisture content and the antagonist action when challenged with other fungi would need further investigation. The second log trial was done to evaluate several isolates from the first log trial at a different time of year (June to November). Although isolates 4650 and 5040 did not perform significantly better than the control in this particular study, isolate 3410 showed significant sapstain control from the control logs. It is not unusual to find several different species of *Ophiostoma* growing in close proximity on a single piece of wood (Seifert, 1993). With this fact in mind and the varied growth properties associated with each albino species developed. The use of treatments containing mixtures of albino *Ophiostoma* species was explored. This field trial showed varied results. No significant reduction in stain was found for any mixture compared to the control but one treatment had reduced amounts of stain.

No albino treatment applied in the field to date was able to successfully stop the colonisation of wild type strains. However, some strains showed potential for the control of sapstain. The future of the use of albino technology to control sapstain in logs in terms of science and commercial benefit relies upon research to determine the conditions to give predictable results and potentially different products for different seasons. A large number of albino strains of *O. piceae*, *O. pluriannulatum* and *O. floccosum* are available from this study that could used in continued field evaluations in New Zealand to control sapstain in *P. radiata* logs and for other bioprocess technologies.

The isolation of sapstain fungi in this thesis used traditional methods of separation into pure culture and subsequent identification using morphological features and mating capabilities. There was a concern that if the isolation of sapstain fungi was not done properly, it could result in unrepresentative data. Some of the sapstain species were obtained in culture more easily than others. There is a concern with overgrowth on culturing media by prolific mould or secondary colonising fungi. In this thesis culturing of *Ophiostoma* species was facilitated by the addition of the antibiotic cycloheximide (Harrington, 1981). To isolate other sapstain fungi not tolerant to cycloheximide, we also used a general medium (malt yeast extract agar) amended with only antibiotics that suppressed bacterial growth (Media 4). Using this media the isolation of *S. sapinea* was facilitated. No species of *Ceratocystis* were encountered in this thesis. *Ceratocystis* species are slow growing on agar media and not tolerant to cycloheximide (Harrington, 1981). Whether this fungus was present but overgrown by other fungi on Media 4, is uncertain. However, many *Ophiostoma* species were also isolated from Media 4, suggesting that it would be possible to isolate *Ceratocystis* species if they were present.

The ability to detect sapstain fungi in wood before substantial stain damage occurs and to differentiate sapstain fungi from common surface moulds is an important goal in wood protection. The traditional methods for detecting fungi in wood are by direct microscopy and isolation into pure culture. Direct microscopy demonstrates the presence of fungi, but rarely allows for the identification of fungal species. Isolation into pure culture is only the first step for the identification of species as mating capability studies and DNA sequence analysis are often required. The methods for unequivocal species identification are time consuming and may take up to six weeks.

This thesis described the development of a potential method to identify sapstain fungi more easily. The use of proteomics was investigated to find unique cell wall proteins from a number of sapstain fungi. The unique protein or proteins were to be partially sequenced and protein sequence data used to develop synthetic peptides, which were to be subsequently used as antigens to produce antibodies.

Immunological methods primarily ELISA (enzyme-linked immunosorbent assays) have been used successfully for detecting plant pathogens including fungal species (Wycoff *et al*, 1987; Dewey *et al*, 1989; Plascencia *et al*, 1996) in herbaceous plants. Breuil *et al* (1988 and 1990) developed an ELISA to detect an unidentified *Ophiostoma* species in artificially inoculated wood using polyclonal antibodies. These immunological assays were able to detect sapstain fungi in wood but were cross-reactive with other fungal species. Banerjee *et al* (1994) produced monoclonal antibodies to *O. piceae* that were less than 5% reactive with other fungi. Chemical and enzymatic modification of the antigen revealed that the epitopes recognised by the monoclonal antibodies were glycospecific. Taxonomic applications using immunological techniques are limited due to the large number of similar antigens shared by closely related species making detection of unique antigens difficult (Breuil and Seifert, 1993). It was believed that by the use of proteomics a unique protein would be identified. In this thesis, it was shown that proteomic analysis of cell wall proteins from a number of sapstain fungi and wood-inhabiting fungi was possible. Using MALDI mass spectroscopy, a peptide mass map of each unique protein spots from two-dimensional gel electrophoresis was obtained. The peptide mass maps were compared to protein databases, but no successful identification of any proteins occurred. Other methods were described in Chapter 7, which could be potentially used to obtain better information on the unique spot found. Unfortunately these options were not available due to budget restrictions. The use of proteomics, however, was shown to be a useful technique to isolate unique proteins.

In summary, the following objectives were identified as possible milestones for future sapstain research, both fundamental and applied (the order does not represent a ranking in terms of importance):

- Examine the effect of melanin production in differing moisture condition, water activity, relative humidity and a more in depth study of the effect of high temperature on melanin production.
- Identify the mechanisms of induction of the melanin genes
- Identify the *Botryosphaeria* species isolated in New Zealand and establish significance to the forest industry.
- Determine the environmental factors effecting the efficiency of albino Ophiostoma strains to control wild type sapstain fungi
- Investigate the metabolite produced by albino *O. floccosum* strains that is effective against *S. sapinea.*
- Explore the use of tandem mass spectroscopy and partial sequencing of the unique proteins from sapstain fungi found in two dimension gels.

In conclusion, this study addressed the importance of sapstain fungi in New Zealand, particularly on *P. radiata*, and increased our understanding of ecological requirements of the different sapstain species. Differences between these species with respect to colonisation and the development of associated stains were identified and investigated, enabling a critical evaluation of those species that were found to be most significant to the New Zealand forest industry. The future of sapstain management and control depends on a thorough ecological understanding of key sapstain fungal species involved in the cosmetic degradation of wood and an ability to manage the impacts of these species on the New Zealand forest industry using a combination of forest management practises and biological and chemical control techniques.

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Appendix 1

Copy of the paper submitted by the candidate, her supervisor and colleagues at the University of Minnesota to the journal "Holzforschung", which is currently in press.

Albino Strains of *Ophiostoma* Species for Biological Control of Sapstaining Fungi.

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Summary

Albino strains of *Ophiostoma floccosum*, *O. piceae* and *O. pluriannulatum* were selected and screened for biological control of sapstaining fungi on New Zealand radiata pine (*Pinus radiata*). Albino strains were obtained through matings and single ascospore isolations from cultures of prevalent species in New Zealand. These strains do not synthesize the melanin-like hyphal pigments of common sapstaining fungi. Additional mating studies were also carried out to obtain isolates of *O. piceae* and *O. floccosum* that lacked pigmented fruiting structures. Several albino isolates of *O. piceae* with colorless synnemata and isolates of *O. floccosum* with little to no synnemata were obtained. Biological control potential of the albino isolates was evaluated in the laboratory by challenging them on wood chips with fungi that cause extensive sapstain in pine, *Leptographium procerum*, *Ophiostoma piliferum* and *Sphaeropsis sapinea*. Many albino isolates of *O. floccosum*, *O. piceae* and *O. pluriannulatum* were effective in stopping the challenge fungi from staining wood chips and were fast growing and colorless when grown unchallenged on wood chips. Selected albino isolates of *O. pluriannulatum* were used in two field trials in New Zealand to control sapstain. Several strains were found to significantly reduce dark sapstain as compared to the untreated control logs.

Introduction

New Zealand produces large diameter radiata pine (*Pinus radiata*) in short rotations, making it the dominant species used in its forest products industry. As with other fast growing pine species, radiata pine produces wood that consists primarily of sapwood, and is susceptible to dark discolorations due to sapstaining fungi. As a result, significant losses are incurred by the New Zealand forest products industry due to these discolorations.

Sapstain, also called blue stain, is caused by pioneer-colonizing fungi, such as *Ophiostoma*, *Ceratocystis*, *Leptographium* or *Sphaeropsis* species that utilize simple carbohydrates, fatty acids, triglycerides and other components of the sapwood (Blanchette *et al.* 1992; Farrell *et al.* 1993; Wang *et al.* 1995). The dark stain produced by these fungi is due to melanin and melanin-like compounds that are localized within the fungal hyphae (Zink and Fengel 1988; Zimmerman *et al.* 1993). As the fungus grows in wood cells, pigmented hyphae impart a discoloration to the wood (Zink and Fengel 1988; Blanchette *et al.* 1992). Sapstain fungi are not thought to compromise strength properties of wood in early stages of colonization, although discoloration decreases the value of wood used for lumber or paper production (Zabel and Morrell 1992; Seifert 1993).

Sapstain has traditionally been controlled with anti-sapstain chemicals; however, toxicity concerns and the environmental effects of many chemicals used have prompted investigations for alternative methods of control. Biological control using albino strains of sapstaining fungi is a new method that can be used. Investigations using colorless strains of *Ophiostoma* species have been successful in controlling sapstain (Blanchette *et al.* 1992; Farrell *et al.* 1993; Behrendt *et al.* 1995a; Behrendt *et al.* 1995b; Schmidt and Müller 1996; White-McDougall *et al.* 1998). By applying a colorless strain of *Ophiostoma* to freshly cut logs the fungus can preferentially colonize the sapwood, thereby capturing nutrient resources and inhibiting subsequent colonization by dark staining fungi. The detrimental effects of sapstaining fungi are also important in the Canadian forest products industry. Surveys recently completed in Canada have identified *Ophiostoma* species as the most prevalent sapstaining organism (Uzunovic *et al.* 1999).

The objectives of this study were to: 1) develop colorless strains from species of *Ophiostoma* prevalent in New Zealand, 2) evaluate selected strains for their potential to control aggressive sapstaining fungi on wood, 3) obtain strains that are completely free of pigment including pigmentation in and around synnemata, and 4) to test selected albino strains in field log trials for their anti-sapstain effect.

Materials and Methods

Colorless Strain Selection

Strains of three common *Ophiostoma* species, *O. floccosum, O. piceae* and *O. pluriannulatum*, isolated from radiata pine in various regions of New Zealand, were used in mating studies. Isolations from stained wood were made by culturing small segments of wood on a semi-selective medium for *Ophiostoma* species amended with cycloheximide (Harrington 1981). Pure cultures were maintained on 1.5 % malt extract agar (MEA).

Matings were completed of each species to produce ascospores for single spore isolations. Tester strains of known mating types were used to determine the mating types of unknown cultures. Matings of *O. pluriannulatum* were carried out by transferring A and B strains to opposite sides of a petri plate containing 1.5 % MEA and allowing them to grow together. Perithecial formation occurred in the center of the plate where the two cultures merged. Matings of *O. floccosum* and *O. piceae* were carried out by inoculating 1.5 % (MEA) amended with several sterile pine twigs and/or wood chips. Media amended with wood was inoculated with one mating type followed by inoculation of the opposite mating type 2-3 days later. Ascospores were harvested 2-4 weeks after matings by dispersing spore droplets into sterile water. A dilute ascospore suspension was streaked onto *Ophiostoma* select medium. Spores were germinated and individually transferred to 1.5 % (MEA). Thousands of single spore isolates were evaluated for mycelium that lacked pigmentation. Selected isolates were incubated at 5 °C for 2-4 weeks. Isolates that remained colorless after this "cold treatment" were tested further in challenge experiments. Selected isolates were also used in additional mating studies to obtain a larger number of colorless strains. Many colorless mycelial isolates of *O. floccosum* and *O. piceae* continued to produce pigmented synemmata, and some isolates of *O. floccosum* produced extracellular pigment that was found around the bases of synnemata. Additional screening was done to select isolates with reduced or no pigmentation in or near synemmata stalks.

Evaluation for Biological Control Potential

Challenge experiments were carried out to evaluate the potential of selected isolates to control sapstain produced by three fungal species isolated from stained radiata pine logs in New Zealand, *Leptographium procerum*, *Sphaeropsis sapinea*, and *Ophiostoma piliferum*. These isolates cause dark staining in wood when inoculated alone. Petri plates containing three sterile pine wood chips (approx. 1.5 X 2.5 mm in size) were inoculated with 0.25 ml per chip of an albino spore suspension (approx. 6 X 10^6 spores/ml) and incubated for three days at 21 °C. Inocula for *L. procerum* and *O. piliferum* were prepared by rinsing plates of growing cultures with sterile water and the spore suspension was used to inoculate the albino treated wood chips. For *Leptographium* species and *Ophiostoma* species, each chip was inoculated with a 0.25 ml spore suspension with a concentration of approximately 6 X 10^6 spores/ml. Since *S. sapinea* does not produce spores in culture, inoculum was prepared by washing plates of growing cultures with sterile water and macerating the mycelium in a sterile blender to an approximate concentration of 2.5 X 10^5 mycelial fragments/ml. Controls for the challenge fungi were inoculated alone on wood chips. After two weeks of growth at 21°C, stained wood chips were scored. A rating of 1 to 5 was used to evaluate the wood and mycelial coloration; 1 = white/non-stained wood, 2 = slight gray, 3 = gray, 4 = dark gray, 5 = black.

Colorless isolates were also grown on pine wood chips to monitor their growth and ability to grow pigment free. Isolates were prepared by rinsing a growing culture with sterile water, and a 5 ml spore suspension at a concentration of approximately 6 X 10^6 spores/ml was used to inoculate the wood chips. After a 14-day incubation period, the isolates could be visually separated using a similar 1-5 rating scale that was used in the challenge study. Categories for growth were included in the 1-5 rating scale. Isolates given a rating of 1 had excellent growth and only white mycelium that completely colonized the non-stained wood chips. Isolates given a rating of 5 exhibited poor growth and/or dark staining.

Field Trials with Albinos on Logs

Field trial 1 was established in mid November 1997 and continued until April 1998 at Kinleith Mill in the Central North Island of New Zealand. Isolates chosen for this field trial were albinos that received the highest ratings in the laboratory challenge experiments. The site for the field trial was an elevated storage site within 400 m of the forest. Six-meter logs cut from trees approximately 24 years old were obtained from the Kinleith Forest and transported to the site the day after felling. Logs were then cut into 4 pieces of 1.5 m each and randomly laid out into piles for treatment.

In Trial 1, seventeen different albino *O. pluriannulatum* strains were inoculated onto logs using a backpack sprayer at a concentration of approximately 1×10^{11} colony forming units per litre. A set of logs sprayed with only water was established as a control. Albino cultures and the water control were sprayed onto nine logs per treatment at a volume of 4 L. After 6 months in the field (November to May, the Austral summer and autumn months), the logs were assessed for coverage of sapstain on four internal surfaces of each log. All logs were sliced into five pieces (30 cm intervals) and each face was assessed immediately after being sprayed with water. The percentage sapstain coverage on each face was estimated by two groups of two assessors each. The assessors estimated the total amount of coverage by blue, gray and black stain at 5% intervals on the entire transverse surface of the wood disks taken from the internal sapwood of the logs, scoring the amount of stain from 0 – 100 %. Statistical analysis was performed as analysis of variance and Tukey's test for comparisons of means using Minitab 12 for Microsoft Windows.

Field trial 2 was established in June 1998. The site and log parameters were the same as trial 1. The 10 logs per treatment were inoculated with the most successful albino strains from log trial 1. They included the albino *O. pluriannulatum* strains 5040, 4650 and 3410 and a control treatment with water.

The albino fungi were prepared by inoculating 10 one-liter flasks with cultures and growing in a shaking incubator at 25 °C for 2-3 days. The excess growth medium was removed by centrifugation and the fungal suspensions were resuspended in 3 L of 100mm Tris HCl buffer at pH 7. Approximately 1×10^{12} colony forming units per albino strain were then resuspended in 50 L of water. A commercial spray unit was used for log inoculation as compared to backpack sprayers used in field trial 1. A 3 log X 3 log replicate block design was used for the trial. After 3 months the logs were assessed for stain development as in the first log trial.

Results

Mating Studies

Single ascospore isolations yielded many strains that had varying degrees of desirable traits, such as reduced hyphal pigments and/or little to no synnemata production. These isolates were then incorporated into mating studies with other isolates of the same species to obtain additional colorless strains with desired characteristics. Thousands of single ascospore isolations were screened to obtain the first colorless isolates of each species. Albino strains of *O. floccosum* had colorless mycelium but retained synnemata with dark pigmented stalks. *Ophiostoma floccosum* had no pigmentation evident in albino mycelium, but a red-brown coloration remained in the synnemata. This pigmentation also extended into the medium around the base of synnemata. Continued mating studies of albino *O. floccosum* isolates produced cultures with no synnemata or with very few synnemata when grown on wood (Fig. 1). Initially selected isolates of *O. piceae* albino strains lacked pigmentation in mycelia but retained black synnemata. Additional mating experiments yielded several albino isolates that had hyaline synnemata (Fig. 2). *Ophiostoma pluriannulatum* does not produce synnemata, therefore additional matings of albino isolates were not necessary with this species. It has been shown in previous investigations using *O. piliferum* that melanin is necessary for perithecial development (Zimmerman *et al.* 1993). Therefore, matings of two albino isolates did not produce perithecia, and cannot be used in mating studies.

The “cold treatment” used to stress cultures and induce pigmentation resulted in pigment production by many isolates originally classified as colorless when grown on culturing media. These cold-stressed pigmented isolates were eliminated from further experimentation. Isolates remaining colorless after “cold treatment” screening were used in the challenge experiments.

Challenge Experiments

Visual observations on wood chips inoculated with albino strains showed white mycelial growth 3 days after inoculation. Two weeks after challenging the colorless strains with the various dark sapstaining fungi, wood chips were categorized into the 1 to 5 rating scale. The results showed that not all albino isolates performed the same when challenged with dark staining fungi (Table 1). Isolates receiving a rating of 1 showed complete control of the dark staining challenge fungi, leaving the wood chips entirely white and non-stained. A rating of 5 showed failure of stain prevention and wood chips were black. Ratings of isolates between 2-4 showed some control in which wood chips were slight gray, gray or dark gray, respectively. Figure 3 shows wood chips inoculated with *O. piliferum* along with wood chips inoculated with a colorless *O. piceae* isolate followed by inoculation with an *O. piliferum* staining isolate. The albino strain successfully colonized the wood and prevented the stain-causing fungus from growing in the wood.

The percent of albino isolates in each rated category after challenging with three different dark staining fungi is shown in Table 1. *Ophiostoma floccosum* and *O. piceae* have a large percentage of isolates with a rating of 1 resulting in excellent control of the three challenge fungi. *Ophiostoma pluriannulatum* had the fewest isolates given a rating of 1, but many of these isolates gave excellent control. Growth of albino single ascospore cultures on wood chips inoculated alone without challenge fungi to test growth characteristics and their capacity to remain colorless, exhibited variations within and among species (Table 1).

Field Trial Results

After 6 months, logs inoculated in the field with 17 different albino *Ophiostoma* strains were assessed. With an ANOVA (analysis of variance) of the data and outliers removed from the mean stain values, log treatments with eight albino *O. pluriannulatum* strains (5040, 3410, 4890, 4650, 5080, 4680, 6110, and 6010) showed significantly less stain than the control logs (Fig. 4). There was a group of albinos that were not significantly different to the control logs and a group (7036, 7014, and 4630) that were statistically more stained than the controls (Fig. 4). Since log diameter varied in this trial, the logs were assessed with regard to diameter and examined for potential confounding effects of sapstain coverage. Log diameter had no influence on the level of discoloration in this trial.

In a second log trial established in June 1998, albino *O. pluriannulatum* strains 3410, 5040 and 4650 from the first trial were again inoculated onto radiata pine logs. This trial showed that albino *O. pluriannulatum* strain 3410 had significant reduction in the amount of sapstain in comparison to control logs (Fig. 5). The other two albino *O. pluriannulatum* strains (5040 and 4650) were not statistically different from the control logs in the amount of sapstain (Fig. 5).

Discussion

These studies show that colorless *Ophiostoma* isolates can be obtained to significantly reduce stain in logs of *Pinus radiata*. Using single ascospore isolations and a series of mating studies, a large number of colorless strains were obtained from different species of *Ophiostoma*.

In the process of developing the colorless strains used in this investigation, cultures of *O. piceae* and *O. floccosum* with varying melanin production have been described. Although the mechanisms of melanin formation are not completely known, this study suggests that different genetic factors are likely to be responsible for overall pigment production in *Ophiostoma*. Pigment production in the vegetative mycelia and pigments produced in and around synnemata required different mating selections to obtain melanin-free cultures. Further studies of these mechanisms could lead to a better understanding of stain production by these fungi. Pathways for pigment formation may be similar between *O. floccosum* and *O. piceae* because of their related phylogeny in the *O. piceae* complex (Harrington *et al.* 2000).

By using *in vitro* screening methods involving cold treatments and challenge experiments, isolates with optimum biological control potential were separated from other less effective cultures. The strains generated in this study are also being screened and utilized for other purposes such as biological pretreatment of wood to remove wood extractives and alleviate pitch problems during pulp and paper production (Farrell *et al.* 2000). Previous studies have shown that albino strains of *Ophiostoma* are capable of degrading wood extractives, including triglycerides, fatty and resin acids (Blanchette *et al.* 1992; Brush *et al.* 1994; Farrell *et al.* 2000; Farrell *et al.* 1993; Wang *et al.* 1995). Degradation of these extractives from wood chips before they are used in the paper making process indicates these strains could have significant application in new bioprocessing technologies as depitching agents.

Melanin production in fungi is thought to be important for resistance to microbial lysis and protection from ultraviolet light and desiccation (Bloomfield and Alexander 1967; Brasier 1978). It also plays a role in perithecial development (Zimmerman *et al.* 1993). But the decreased amount or lack of melanin in the albino strains obtained in this study does not appear to inhibit the aggressiveness or growth characteristics of the fungi. The laboratory challenge experiments demonstrate that albino strains of *Ophiostoma* can be effective at preventing stain occurring from different species of sapstaining fungi. In addition to an isolate of *O. piliferum* that caused dark stain, New Zealand isolates of *L. procerum* and *S. sapinea* that also cause dark stains were inhibited. These results support previous findings (Behrendt *et al.* 1995a; Behrendt *et al.* 1995b; Müller and Schmidt 1995; Blanchette *et al.* 1997) that showed some success using albino strains to control sapstain in preliminary experiments. Concern that biological control using one organism may not sufficiently control many different species of stain fungi has been raised (Kang and Morrell 2000). Results presented here demonstrate a single albino strain can control several different genera of stain fungi that are commonly found in New Zealand timber products (Farrell *et al.* 1997).

The studies described in this paper provide important new information on the ability of laboratory selected albino strains exhibiting superior biocontrol potential to be used to reduce sapstain on radiata pine in New Zealand. This research also demonstrates that the most prevalent and aggressive *Ophiostoma* species native to a specific country can be used to obtain albino strains for biological control. This avoids the problems associated with introducing foreign strains of fungi into areas that they are not native.

The effectiveness of controlling sapstain varied among strains tested in field trials. The second log trial was done to evaluate several isolates from the first log trial at a different time of year (June to November). Although isolates 4650 and 5040 did not perform significantly better than the control in this particular study, isolate 3410 showed significant sapstain control from the control logs. Strains 4650 and 5040 may not have performed as well as in trial 1 due to prior contamination of logs with wild type sapstaining fungi during harvest and transport. Another possibility is that these strains are not ideally suited for the environmental conditions occurring at the time of the study. Additional field experiments are warranted to evaluate more albino strains. In this study, no attempt was made to optimize field inoculation procedures or methods to insure comprehensive coverage of the debarked logs by the albino strains. The time of inoculation by the biocontrol agent is crucial and should be done immediately after cutting (Behrendt *et al.* 1995b). Additional field trials are needed to elucidate the crucial environmental factors that could affect the success of the albino strains and result in more effective control. Biological control of sapstain over a six-month period is also an exceedingly long time for the albino strains to remain efficacious. Previous investigations indicate that albino strains performed well as biocontrol agents over a shorter period in field studies (Behrendt *et al.* 1995a; Behrendt *et al.* 1995b). Logs used in New Zealand field trials were completely debarked, which resulted in the entire circumference of the logs being exposed. This large area of exposed sapwood and inadequate inoculum coverage may have contributed to a reduced effectiveness of sapstain control. Improved methods of inoculation and application could provide better coverage and better adherence to the logs. When using biocontrol agents, the timber industry also may have to accept shorter periods of log storage (2-3 months instead of 6) and more rapid processing of logs treated with biocontrol fungi.

The results presented demonstrate that biological control using albino stains of Ophiostoma can be successful in New Zealand but more research investigation is needed to optimize these biological control processes. A large number of albino strains of *O. piceae*, *O. pluriannulatum* and *O. floccosum* are available from this study and can be used in continued field evaluations in New Zealand to control sapstain in radiata pine logs and for other bioprocessing technologies.

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Table 1. Percent of albino strains of *O. piceae*, *O. floccosum* and *O. pluriannulatum* rated for biological control potential when challenged against wild type strains of *L. procerum*, *O. piliferum* and *S. sapinea*. Albino strains listed as not challenged were rated for growth and ability to grow free of pigment on wood chips. 1 = white/non-stained wood, 2 = slight gray, 3 = gray, 4 = dark gray, 5 = black

Strain	Rating for biological control potential and stain production				
	1	**2**	**3**	**4**	**5**
O. piceae					
L. procerum	59	26	10	4	1
O. piliferum	82	13	5	0	0
S. sapinea	70	26	3	0	0
No challenge	63	12	25	0	0
O. floccosum					
L. procerum	90	10	0	0	0
O. piliferum	92	6	1	0	0
S. sapinea	75	21	4	0	0
No challenge	48	14	6	8	24
O. pluriannulatum					
L. procerum	8	34	43	15	0
O. piliferum	55	10	30	4	0
S. sapinea	11	16	30	43	0
No challenge	22	35	24	19	0

Fig. 1. Culture of a naturally occurring *Ophiostoma floccosum* (left) and an albino culture of *O. floccosum* (right).

Fig. 2. Typical synemma of *Ophiostoma piceae* (left), stunted synemma of an albino isolate (center) and a hyaline synemma of an albino isolate (right).

Fig. 3. Wood chips inoculated with an albino strain of *Ophiostoma piceae* followed 3 days later by a staining isolate are shown in the top row. No stain is present in these wood chips. Bottom row of darkly stained wood chips were inoculated with a staining isolate of *O. piliferum*.

Fig. 4. Visual evaluation of sapstain on logs from field trial 1 treated with *O. pluriannulatum* albino strains and control logs receiving only water with outlying data points removed. Treatments with an * had significantly less ($p=0.05$) stain as compared to the control.

Fig. 5. Visual evaluation of sapstain in logs from field trial 2 showing the mean percentage of sapstain coverage for treatments. Treatment with an * had significantly less ($p=0.05$) stain than the control.

Figure 1.

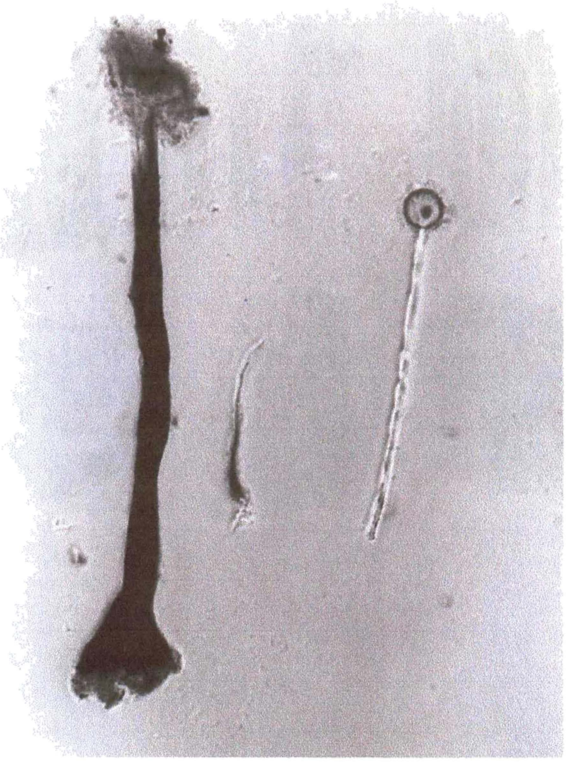

Figure 2.

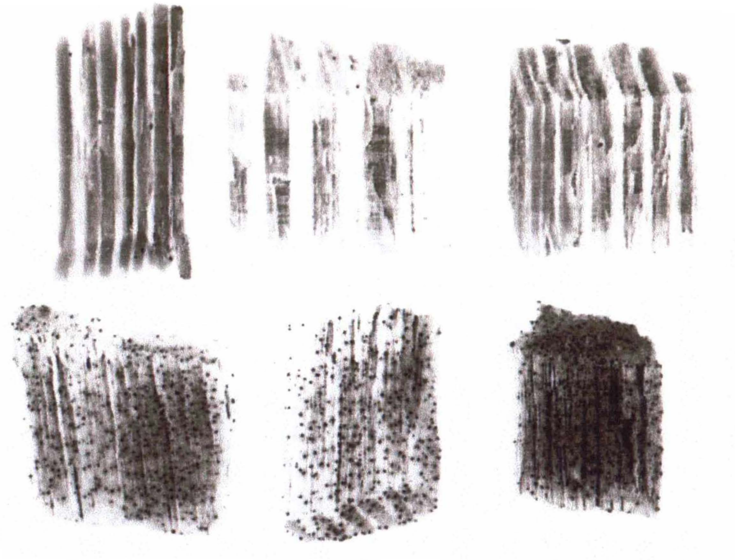

Figure 3.

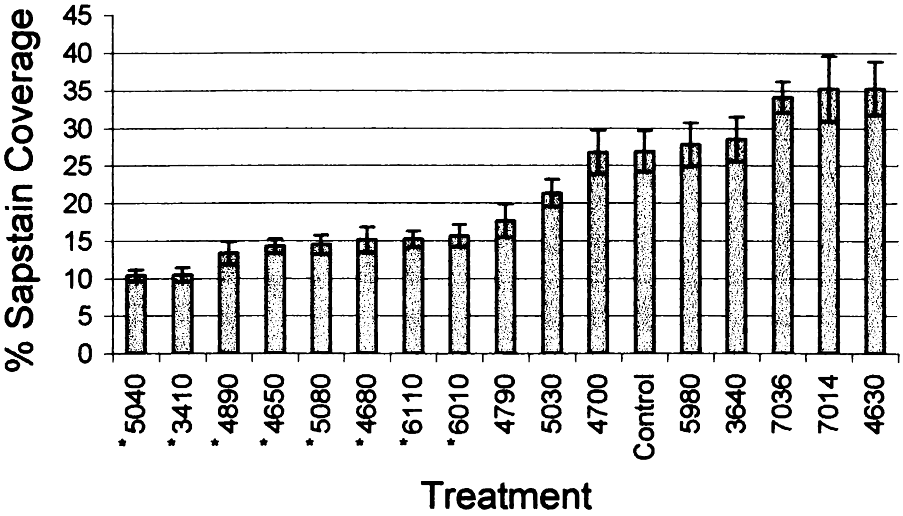
% Sapstain Coverage
45
40
35
30
25
20
15
10
5
0
*5040
*3410
*4890
*4650
*5080
*4680
*6110
*6010
4790
5030
4700
Control
5980
3640
7036
7014
4630
Treatment

Figure 4.

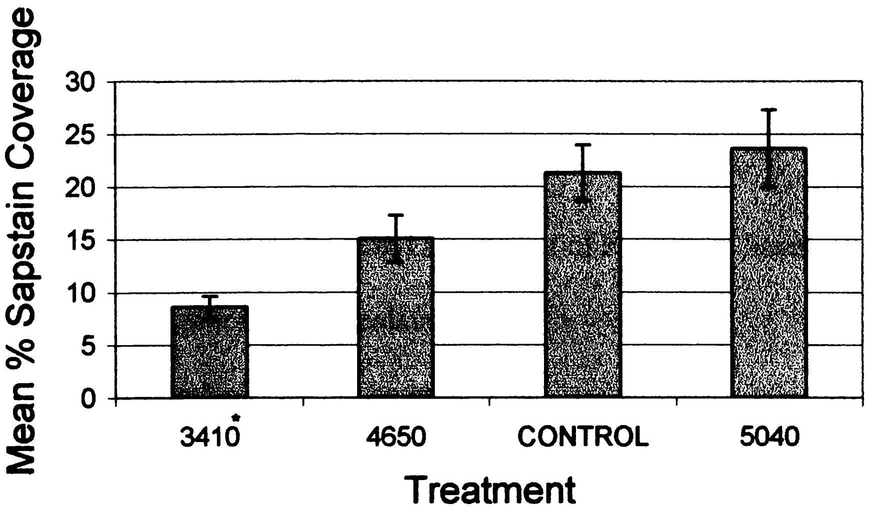
Mean % Sapstain Coverage
30
25
20
15
10
5
0
3410*
4650
CONTROL
5040
Treatment

Figure 5.

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A PROPOSED RAPID METHOD FOR DETECTION OF SAPSTAIN FUNGI IN RADIATA PINE

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Abstract Sapstain is the bluish-black discolouration in logs and timber caused in New Zealand by two classes of fungi, *Sphaeropsis sapinea* and a variety of Ophiostomataceae. The nature and economic importance of these staining fungi has prompted research into the development of quick, specific, sensitive, and inexpensive assays to detect and quantify fungal infections of wood. Development of antibody assays for *S. sapinea* and the major *Ophiostoma* strains fulfils most of these requirements.

Firstly, a survey of the major sapstaining species in Kinleith Forest and mill sites has been done to establish the major contributors to sapstain in this forest area. The major sapstain species have been used to develop the antibodies that will be used for the final detection assay.

Antigens have been obtained from partially purified cell wall glycoproteins. Polyclonal and/or monoclonal antibodies will be developed in either sheep or mice respectively. Enzyme-linked immunosorbent assays will be established with the antibodies to detect sapstaining fungi in wood.

The presentation discusses the practicality of Sapstain Antibody Detection for use in the forest, at mill sites and for marketing purposes.

Introduction

Growth of sapstain fungi on radiata pine is an important economic problem for the New Zealand forest industry, due the production of a bluish, black discolouration by the fungi in the wood and wood products. The cost of sapstain for the forestry industry is due to the downgrading of products, in credits to customers for non-specified product delivered and opportunity costs lost due to failure to meet market needs. To control wood discolouration, fungicides are commonly applied that can have concomitant environmental problems (Behrendt *et al.*, 1995).

Causative agents of sapstain in New Zealand were studied in the late 1960s and 1980s (Butcher, 1967, Hutchison and Reid, 1988). Both these researchers sampled only a few areas of the North Island of New Zealand. The organisms identified were as follows: *Sphaeronaemella pinicola*, *Ceratocystiopsis falcata*, *Ophiostoma piceaperdum*, *O. ips*, *O. novo-zealandiae*, *C. piceae*, *C. coronata*, *C. rostrocoronata*, and *C. piliferum*. In 1996, Farrell *et al.* (1998) at Waikato University started a survey of the sapstaining fungi in New Zealand. This survey has shown that there are at least 20 different sapstaining fungi in New Zealand, including *Sphaeropsis sapinea* and members of the Ophiostomataceae family.

The ability to detect sapstain fungi in wood before substantial damage has occurred is an important goal in wood protection. The traditional methods of detection of fungi in wood are direct microscopy and isolation into pure culture (Breuil *et al.*, 1988). The first method of detection demonstrates the presence of fungi, but rarely allows for identification of fungal species (Breuil *et al.*, 1988). Isolation into pure culture is only the first step for identification of species, as mating compatibility and DNA sequence analysis are often required. The method for unequivocal species identification is time consuming and may take up to six weeks.

Another method of detection of fungal species is by antibody technology. Antibodies are produced in an animal in response to the introduction of foreign matter, the antigen. Successful antigens include proteins, carbohydrates, nucleic acids, lipids and many other naturally occurring or synthetic compounds (Harlow and Lane, 1988). The antibody is obtained from the animal and used in conjunction with an assay method to detect the chosen antigen. Two types of antibodies can be produced: polyclonal and monoclonal antibodies.

Polyclonal antibodies are developed by injecting an antigen into the chosen animal in several dosages. The polyclonal antibodies are then purified from the raw serum fraction of the blood. They represent a complex array of antibodies

Table 1. Sapstain fungi isolated from Kinleith Forests during a two-year survey.

Sapstain Fungus	**Numbers of isolation**
S. sapinea	83
O. quercus	47
O. ips	44
O. piliferum	34
O. floccosum	23
Pesotum cupulatum	12
Leptographium procerum	7
O. piceae	7
O. pluriannulatum	4
O. huntii	1
O. coronatum	1
O. stenoceras	1

isolated and identified in the Kinleith area and *O. floccosum* was ranked fourth, but was the most commonly found *Ophiostoma* species in New Zealand as shown by Farrell *et al.* (1998).

The fungi chosen for antigen production and determination were selected due to their abundance in the Kinleith Forest area. No documented antibody production has been performed on the following *Ophiostoma* species selected: *O. ips*, *O. floccosum*, and *O. quercus*. Antibodies have been produced from *S. sapinea* originating in New Zealand (Singh, 1999), but these antibodies showed some cross-reactivity with other *Ophiostoma* species and other fungal species (Singh, 1999). To develop polyclonal antibodies that are less cross reactive with other fungal species, more development at the antigen level was needed.

Concerns about the impact of fungicides on the environment and high trreatment costs against sapstain fungi in logs and lumber have led to the need for sapstain prediction methods and models. In the early stages, colonisation of unseasoned wood by sapstain fungi can proceed without obvious symptoms. Mould and other fungal surface stains on logs and lumber have been confused with sapstaining fungal growth. An attractive prospect is the use of ELISA or other antibody type testing to detect the onset of sapstain colonisation in sufficient time to allow the successful application of fungicides or other control methods.

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THE INTERNATIONAL RESEARCH GROUP ON WOOD PRESERVATION

Section 1 **Biology**

Future Directions for Biological Control & BioActivity®

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Department of Biological Sciences, The University of Waikato, Hamilton, New Zealand

Paper prepared for the 32nd Annual Meeting
Nara, Japan
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2

Future Directions for Biological Control & BioActivity®

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Future directions for biological control of deleterious organisms on wood, and commercial market success, are dependent upon efficacy, cost and shelf life in comparison to other available means, and appropriate fulfilment of registration requirements. Basic and applied research involving ascomycete and/or basidiomycete fungi are described regarding solid wood applications of biocontrol of sapstain, and pulp and paper applications of pitch removal. Crucial to the efficacy assessment for the biological control agent is methodology to determine deterioration and diagnostics.

Critical parameters that affect biological control efficacy in the field from our experience are as follows:

1. Cause of the deleterious condition, including presence of deleterious organism(s) and/or the environment in which they thrive.
2. Biochemical activity and growth characteristics of the biological control agent, what we call BioActivity.
3. The wood resource and the environment in which it needs to be controlled.

Keywords: Biological Control, biopesticides, ascomycete, sapstain, registration

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Future Directions for Biological Control & BioActivity®

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Biological Control Definitions

Biological Control is a broad term, and in its broadness lies partly the confusion as to its scientific basis, efficacy and commercial market success and future potential. In this paper, we confine the topic of biological control to the substrate wood as logs, timber or chips, and type of control desired as prevention of deterioration as caused by microorganisms. Deterioration is defined as either structural, caused by wood decay organisms, most specifically white- and brown-rot fungi, or cosmetic, caused by the 'sapstain' organisms; the paper will focus on the latter. Biological control, in addition, has implications towards market acceptance regarding biosecurity. We define biosecurity as the concern of importing countries for their biological security such that imported materials do not contain "hitchhiking" deleterious organisms that threaten their agricultural, horticultural, forest industries, or environment. With biological control there is a "reduction of inoculum density or disease producing activities of a pathogen or parasite in its active or dormant state, by one or more organism, accomplished naturally or through manipulation of the environment, host or antagonist, or by mass introduction of one or more antagonsists" (Baker and Cook, 1974). For biosecurity it may be much more desirable to target the organisms on wood with a known and accepted biological whether than undefined organisms (For background on biosecurity, a good source is U.S. Department of Agriculture Importation of Logs, Lumber, and other Unmanufactured Wood Articles, Final Supplement to the Environmental Impact Statement, May 1998).

Biological control is achieved by an organism(s) acting to prevent the deleterious effect of other organisms by antagonism, by antibiosis and/or by competition or any combination of mechanisms. Antagonism is the suppression of growth, survival and/or activity of an organism by another organism(s). The antagonist may only suppress the growth, but in order for the antagonist/biological control agent to be effective, the antagonized organism must not recover and be deleterious during the time in which control is desired, in some circumstances a requirement of years. Some antagonists actively attack the deleterious organism, or consume it for their own nutrition (Jeffries, 1995). Most efficient control by the antagonist may be killing of the antagonized organism by toxins or hyperparatism, but this is not always necessary nor desirable, particularly if there is any lack of specificity. Competition, possibly involving hyphal interactions, occurs to some extent in connection with primary resource capture and is almost always involved in secondary resource capture (Holmer and Stenlid, 1993). The distinction between primary or secondary resource capture is the fungus establishing itself in the absence of competitors (Rayner and Boddy, 1988) or involving the replacement of organisms already present (Redfern and Filip, 1991). The major objectives of most of the last decade of work on biological control have been (1) to identify potential antagonists and treat wood for exposure to wood-attacking fungi, (2) to investigate mechanisms of antagonism, (3) to improve the ability of the antagonist to colonise wood, and (4) to evaluate delivery systems (Highley et al 1994). In this paper we will discuss particularly the work we and our collaborators have carried out relevant to these four points.

Excellent work has been carried out on the use of natural products to control deleterious fungi or insect vectors, though these strategies will not be discussed in this paper. The reader is directed to the demonstration of the potential of essential oils, particularly pine oil based treatments, in controlling sapstain organisms in New Zealand on radiata pine logs and sawn timber at mill sites (Hill *et al* 1997).

We define the circumstances where biological control agents are appropriate to consider for use where there are not highly effective chemical agents available either in terms of efficacy, cost or environmental appropriateness. Biological control agents (BCAs) fit well when they can be used as follows (Harman, 2000): Replace chemical pesticides lost to regulatory action or pest resistance and for which there are no chemical replacements, Replacement, or reduction of use, of chemical pesticides in sensitive environments, Applications where biologicals accomplish tasks not possible for chemical pesticides, and Organic applications. The use of compatible chemical and biological control agents, in order to have synergistic or augmentative control, has not been studied enough, and will require either that the biological control agent be resistant or tolerant to the chemical agent, or that they be applied sequentially such that they are not directly in contact i.e. the biological control agent penetrates into sapwood ahead of the chemical agent's topical application. Interesting research with this type of approach either with essential oils or biocides and biological control agents shows promise (Mcafee and Gignac, 1997; Dawson-Andoh et al 2000).

New Zealand and Introduction to Sapstain

New Zealand is well known for producing radiata pine (*Pinus radiata*) in short rotations, making timber and wood products an integral part of New Zealand's economy (Kininmonth, J. 1991). As with other fast growing pine species around the globe, radiata pine produces wood that consists primarily of sapwood, and is susceptible to dark discolorations due to sapstaining fungi. Sapstain, also called blue stain, though it can be a blue, black, grey, red or brown discolouration, is caused by pioneer colonizing fungi, such as *Ophiostoma, Ceratocystis, Leptographium or Sphaeropsis* species. These fungi are not capable of degrading cellulose or lignin, but metabolise resin extractives, fatty acids, resin acids, and triglycerides, starch and simple carbohydrates, and other components of the sapwood, growing in the ray parenchyma cells, within resin canals, within tracheids and fibre cells and penetrating simple and bordered pits; occasionally forming bore holes through wood cell walls (Blanchette et al 1992; Farrell et al 1993). The dark stain produced by these fungi is due to melanin and melanin-like compounds that are localized within the fungal hyphae (Bell and Wheeler, 1996; Zimmerman et al, 1993). As the fungus grows in the wood cells, pigmented hyphae impart a discoloration to the wood (Blanchette *et al.* 1992). Sapstain fungi are generally believed not to compromise strength properties of the wood, but cause a decrease in wood quality used for lumber or paper production.

Harman, 2000 discussed "Dogmas and Myths" of biological control for agriculture; some of these dogmas and myths have also been published as applicable to biocontrol of wood deterioration but we will show that at least the first three are not necessarily so. Four of Harman's Dogmas are as follows:

1. Biological control agents (BCAs) are necessarily less effective and reliable than chemical pesticides.
2. Single BCAs cannot be effective in diverse environments, on different crops, or against a range of plant pathogens. As a corollary, mixtures of BCAs will be required for successful

long-term control, since individual components colonise different crops, are adapted to different environments, or have different functions.

3. BCAs have simple mechanisms of action that are controlled by one or a few genes and gene products.
4. Registration of BCAs with the U.S.EPA is relatively fast, inexpensive and simple.

Efficacy assessment for biological control

The methodology for assessment of deterioration is crucial to judging success or failure, but it must be rigourous, and conform to international standards. As Tucker et al have written "Essential to the continued development of biocontrol systems is the need for common methods for screening and efficacy testing of biocontrol agents" (Tucker et al, 1997). We believe there is an inherent difficulty in reliance on lab assays which are only indicative and do not represent the full ecological scope in which the biological control agent must demonstrate efficacy. At best, a negative result in an assay in the lab will indicate what won't work in the field. The only tests that are fully reliable are field tests on the appropriate substrate, which one wants to biocontrol; in New Zealand, this is most often mature radiata pine logs, with diameters of 400 – 600 mm, or timber. Therefore international standards of testing must allow for biological control product candidates to be tested under the situation(s) where commercial use is targeted.

Critical parameters affecting biological control efficacy in the field

Causal Organisms of the deleterious condition in New Zealand

When we began our programme in New Zealand in1996, based on our earlier experience with inoculating fungi onto wood for pulp and paper applications (Farrell et al 1992; Blanchette et al 1992), we believed critical to biocontrol efficacy was the understanding of causal organisms on the wood which one wants to control, and adjusting the biocontrol agent, its dose, and/or its application appropriately. "The lack of knowledge about the organisms causing timber discolorations (or stain) posed relatively few problems" while sodiumpentachlorophenol was in use" wrote Kang and Morrell, 2000. Lack of substantive knowledge of the stain fungi has been suggested as inhibiting the development of alternative, nonchemical, stain-prevention methods, such as biological control (Freitag et al 1991).

Studies of sapstain organisms in New Zealand were conducted by two groups in the late 1960's through 1980's. These studies focused on a few selected areas, mainly in the North Island of New Zealand and on material from radiata pine (Hutchison and Reid 1988a,b; Butcher 1968; Butcher and Howard 1968). Our nationwide survey was conducted to broadly survey and identify sapstaining organisms in New Zealand (Farrell et al 1997; Kay *et al* 1997). To date 21 sapstain species have been identified using morphological, cultural, with both non-selective and selective media for isolation, mating and molecular studies, so as not to prejudice the types of organisms found (Farrell *et al* 1998; Harrington et al 2001). The majority of species identified were members of the Ophiostomataceae although the numerically dominant species was *Sphaeropsis sapinea (Diplodia pinea)*; the next most common fungi identified as causing sapstain on radiata in New Zealand were *Ophiostoma floccosum* and *O. ips*. Ophiostomatoid genera include *Ophiostoma, Leptographium, Pesotum* and *Sporothrix*. Whilst there was a degree of seasonal, geographical and site variation in the numerical and species composition within the sapstain population all species were found throughout the country year round. For biocontrol efficacy the staining organisms to be controlled must be those that dominate the wood. Though *S. sapinea* isolates dominate in the forest and freshly harvested wood, they are rapidly outgrown by the Ophiostomataceae, as evidenced by dominance of the Ophiostomataceae at the mill sites, and as isolated from export wood (Farrell et al 1997); the reasons for *Ophiostoma species* dominance we have found are first, outcompetitiveness by faster growth rates in sapwood than *S. sapinea* and second, with some *Ophiostoma species* antibiosis towards *S. sapinea*. Lastly, with regard to causal organisms of sapstain, fungi that infect the harvested logs or timber may or may not be effectively controlled with agents that are topically applied, but endophytic organisms, those which exist in the sapwood in the living tree, are not susceptible to agents that can not penetrate into the sapwood. We are presently completing a three year study of endophytes of New Zealand radiata pine, as well as those of Monterey pine from the Monterey California peninsula, in order to further identify requirements for anti-sapstain treatments (McNew, Harrington, and Farrell, unpublished).

Causal environment in which deleterious organisms thrive on wood

Fungal organisms rely on their presence, the environment (including conditions of appropriate moisture, temperature, pH) and nutrition in order to colonise wood and become established. The Sapstain Danger Index was designed to give a proposed number of days between the felling of a log and when anti-sapstain treatment should be applied, whether the treatment was a biocontrol or chemical agent prior to infection with sapstain fungi. The Sapstain Danger Index (SDI) monitors climatic variables to determine when conditions are ideal for fungal growth and therefore when logs are more likely to become infected with sapstain fungi and they rapidly grow but does not monitor the direct infection of logs. From 1998 to 2000 a study was conducted in Central North Island New Zealand to determine whether or not the Sapstain Danger Index accurately predicted when a log should be treated for sapstain (Cooper et al, 2000). This study was conducted over a wide range of site conditions and seasonal variation and has shown that at two of the eleven sites tested, the Sapstain Danger Index accurately predicted when the logs should be treated for sapstain and at the other nine sites tested the Sapstain Danger Index predicted the number of days between felling and when the logs should be treated for sapstain within two days of infection. Therefore as is the Sapstain Danger Index is close at predicting when felled logs should be treated for sapstain, but is not accurate enough to effectively minimize economic loss caused by sapstain, more research into the factors influencing the rate of infection of logs by sapstain fungi needs to be investigated before commercial reliance on SDI can be implemented.

Physical parameters of wood are often considered as to their relative importance with regard to biocontrol, particularly the ability of the biocontrolling agent to penetrate and establish itself regarding size. Log diameter varied in the New Zealand albino fungi trial (discussed in the next section), and therefore the logs were assessed with regard to diameter and examined for potential confounding effects of sapstain coverage. Log diameter had no influence on the level of discoloration in this trial.

The ability of the biocontrol agent to establish itself on the wood is dependent upon its ability to capture nutrients. We have studied in depth the metabolism of extractives, starch, and carbohydrates in wood as a result of developing methods of combating resin problems in pulp and paper production [for a review of the enzymatic methods developed by Hata and his colleagues of Nippon Paper Industries and fungal methods developed by Farrell and colleagues see Farrell et al, 1997]. From this work we have developed guidelines for successful inoculation of fungi onto wood (Farrell et al 2000) which includes producing a fungal inoculum from growth media in which the organism is expressing the enzymes of choice and which allows it to be freeze dried with viability (shelf life) for over six months, an efficient spray system to cover the wood for good inoculation and which does not decrease fungal viability, and a dosage rate of fifty tonne of wood sprayed with 2 cubic meters water/fungi at a $10^6 - 10^7$ colony forming units of fungi per millilitre suspension; thus, each square millimetre of surface is sprayed with 0.725 to 7.25 fungal cells. Holmer and Stenlid have also discussed the importance of inocula size for successful competition (1993).

Biological Control Success Stories

There are hundreds of papers demonstrating biocontrol in lab assays, or on sterile wood. The critical parameters for understanding individual control agents though limits the development of appropriate screening methods, and we agree with Bruce's comment that with this lack of understanding "it is impossible to enhance the control abilities of selected biological control agents" (Bruce 1992). Though some of these papers show interesting science, and may eventually aid in understanding the mechanisms of biological control, only field demonstration can prove biocontrol. Though this paper focuses on competitive colourless fungal strains with which we have been involved with Robert Blanchette's group at University of Minnesota and others, there has been successful biocontrol demonstrated by the yeast *Pichia sp.* on *Pinus sylvestris* L. (Payne et al 2000), *Gliocardium roseum* on hemlock and fir (Mcafee and Gignac, 1997; Dawson-Andoh et al 2000), *Phialemonium curvatum* on *Populus tremuloides* (Hiratsuka and Chakravarty, 1999), basidiomycetes on a variety of wood species (Blanchette et al 1996) and *Gliocladium viride, Trichoderma hamatum, T. harzianum, Trichothecium roseum*, on radiata pine (Kay et al 1995; Vanneste, 2001).

Albino *Ophiostoma species* as biocontrol agents

Investigations using a colourless strain of *Ophiostoma piliferum*, showed these organisms to be successful in North America to control sapstain principally showing success on *Pinus resinosa* and southern yellow pine (*Pinus sp.)* (Blanchette *et al.*, 1992, Farrell *et al.*, 1993, Behrendt et al., 1995 a,b). Per the aforementioned objective for biocontrol (to improve the ability of the antagonist to colonise wood) these colourless strains were selected using classical genetics, not mutagenesis nor genetic engineering so as not to compromise concerns for field release, to outgrow strains isolated directly from wood, and to achieve greater levels of metabolism of wood nutrients (resin extractives) (Brush et al, 1994; Farrell, et al 1997). It is this property, we term it BioActivity® that makes these strains more efficacious and unique than other fungal isolates in biocontrol applications. These studies demonstrated that applying a colourless strain (obviously the lack of colour, or pigmentation/melanin, the albino characteristic was also a necessary requirement for anti-sapstain effect) of *Ophiostoma* to freshly cut logs allowed the inoculated strain to preferentially colonize the sapwood thereby capturing nutrient resources and inhibiting subsequent colonization by organisms that would occupy the same ecological niche such as the dark staining fungi. Melanin production in fungi is thought to be important for resistance to microbial lysis, protection from ultraviolet light and desiccation. It also plays a role in perithecial development (Bloomfield and Alexander 1967, Brasier 1978, Zimmerman *et al.* 1995). The decreased amount or lack of melanin in the albino strains obtained in this study does not appear to inhibit the aggressiveness or growth characteristics of the albino fungi. Also, from this work we refer to Harman's third Dogma that multiple genes control the biocontrol effect as it is dependent upon multiple activities including but not limited to metabolism, growth, nutrient capture and assimilation.

Schmidt and Mueller in Germany have shown similar success with the same strain of the colourless *O. piliferum* on ***Pinus sylvestris*** sawn timber, and debarked logs controlling sapstain and rot species (Schmidt and Mueller, 1996; Mueller, PhD Thesis, 2000).

Albino isolates of *O. floccosum*, *O. piceae* and *O. pluriannulatum* were effective in field trials in New Zealand on mature radiata pine logs to control sapstain. Isolates chosen for this field trial were albinos, which received the highest ratings in the laboratory challenge experiments. After 6 months in the field, from November to May, the Austral summer and autumn months, the logs were assessed for coverage of sapstain on four internal surfaces of each log. The assessors estimated the total amount of coverage by blue, gray and black stain at 5% intervals on the entire surface of biscuits taken from the internal sapwood of the logs, scoring the amount of stain from 0 – 100%. Statistical analysis was performed as analysis of variance and Tukey's test for comparisons of means using Minitab 12 for Microsoft Windows. Seven albino strains were found to significantly reduce dark sapstain, caused by numerous sapstain organisms, as compared to the untreated control logs and compared to other albino strains which only showed some inhibition of stain and some which showed no inhibition of stain, though all of the albino isolates were shown to be effective in the lab assays (Figure 1). Therefore a single albino strain can control several different genera of stain fungi that have been reported to occur in high frequency in New Zealand timber products (Farrell *et al.* 1997), and be better than a commercial chemical biocide, addressingboth Harman's dogma and previous concerns that biological control using one organism may not sufficiently control many different species of stain fungi (Kang and Morrell, 2000). It is crucial to do the research and field work to find the albinos strains that work best for biocontrol of sapstain on the wood species of interest in the field.

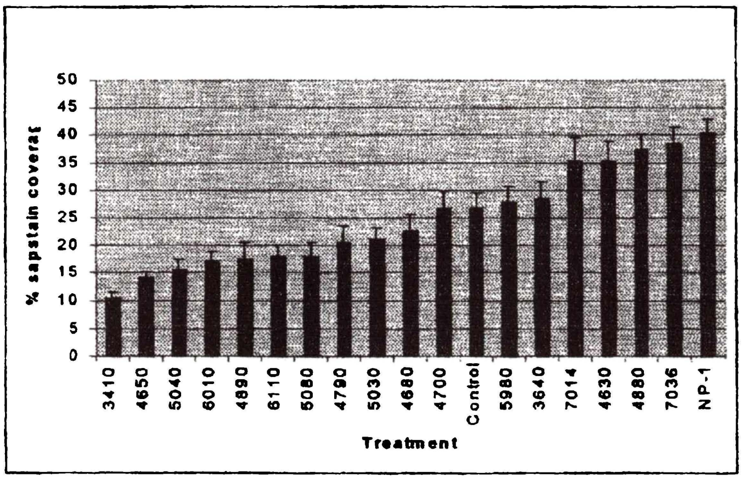

Figure 1 Visual evaluation after six months field trial of sapstain on radiata pine logs treated with albino *Ophiostoma sp.* strains (each of the four digit numbers represents an independent albino strain) and control logs receiving only water and NP1 treated logs (Held et al 2001).

Registration and Approval

For biological control agents, appropriate registration must be carried out in the country of use, and for export-treated wood to determine if further regulatory approvals are required for acceptance in the receiving destination(s). Our experience with colourless *Ophiostoma species* is also that health and safety data are required, such as levels if any of dermal irritation, oral toxicity, eye irritation and fish and avian toxicity, and proof that the biological control agent is found in the locality in which it is to be used, by identification, mating compatibility and molecular data,. Migration studies are often also required to demonstrate potential spread of the organism, and/or lack of appearance of the organism or its biochemicals (i.e. proteins, secondary metabolites) in final products. Diagnostics are extremely helpful for fulfilling these requirements, as well as basic and applied research for efficacy determination of biological control agents, and there has been signicant advances in recent years with serological and DNA-based assays in this area (Breuil et al 1992; Jellison and Jasalvich, 2000; Schroeder et al 2000; Thwaites and Farrell, 2000; Harrington et al 2001).

New Zealand

To determine specific requirements for registration in a country, we depend upon expert advise and the ideal person for advise is one who has both expertise in law and biocontrol – Stephen Parker is just such a person and has kindly provided the following information for those interested in New Zealand and Australia. The requirements for marketing approval of a biological control agent (BCA) in New Zealand have recently changed. Previously, marketing approval for agricultural compounds such as pesticides, animal remedies, fertilizers, etc was regulated under their respective Acts, namely the Pesticides Act 1979, the Animal Remedies Act 1967, Fertilisers Act 1960, etc. As of 2 July 2001 the regulatory environment for agricultural compounds changed. A number of Acts including the Pesticides Act 1979, the Animal Remedies Act 1967, the Fertilizers Act 1960, and the Toxic Substances Act 1979, were repealed. These Acts were replaced by the "partnership legislation" comprising the Agricultural Compounds and Veterinary Medicines Act (ACVM Act) and the Hazardous Substances and New Organisms Act (HSNO Act). The ACVM Act is administered by the ACVM Group of the Ministry for Agriculture and Food (MAF) (http://www.maf.govt.nz/ACVM/). The HSNO Act is administered by the Environmental Risk Management Authority (ERMA) (http://www.ermanz.govt.nz/).

Prior to commencement of the ACVM Act and the HSNO Act, registration of a pesticide under the Pesticides Act required the applicants to provide data associated with the manufacture and use of the product. These data related to identification, manufacture, safety, efficacy, toxicity, environmental toxicity, and residues. Under the Pesticides Act, regulatory interest was triggered by the proprietor representing the product as having a pesticidal use. Under the ACVM Act the definition of the products which fall within its ambit is much broader and seeks to capture all products previously regulated as pesticides, animal remedies, fertilizers, etc. The statutory definition of agricultural compound is based on how the product is actually used, rather than how it is represented for use by the proprietor. The term "biological compound" includes viruses, mycoplasmas, or other microorganisms, whether living or not. The ACVM Act clearly encompasses the regulation of microbial BCAs used as agricultural compounds.

The ACVM Act approaches the regulation of agricultural compounds by classification. Agricultural compounds are placed in classes of product. Some of these classes will be exempt from the requirement for registration under the Act, others will be exempt from the requirement for registration with conditions, others will be required to be registered and require an application for registration to be submitted. Classification, and hence the information required to be supplied by the applicant in order to achieve compliance with the Act, is based on the risks a particular agricultural compound presents. The relevant risk areas under the ACVM Act are risks to trade in primary produce; risks to animal welfare; and risks to agricultural security. The purpose of the Act is also to ensure the use of agricultural compounds does not result in breaches of domestic food residue standards.

The commencement of the ACVM Act represents a move away from the "prescriptive" regulatory environment of the Pesticides Act. The ACVM Act has the potential to provide a more amenable regulatory environment for the registration of BCAs. It is anticipated that at least some BCAs will be classified exempt from registration subject to the condition that their manufacture complies with a standard prescribed under the Act. An area of uncertainty is how residues of a biological control agent and the risk these residues pose to trade in primary produce will be addressed under the ACVM Act. Indeed it remains to be clarified in many jurisdictions what is a "residue" of a BCA. It will be noted that the ACVM Act, unlike the Pesticides Act, does not require a demonstration of efficacy to satisfy the requirements for registration. The validity of claims concerning efficacy of an agricultural compound are to be addressed under consumer protection legislation. An exception to this would be where the efficacy of an agricultural compound was directly relevant to the management of risk in a particular risk area. E.g an agricultural compound claiming use as a fumigant would pose a risk to agricultural security if it was not effective. It will also be noted that the ACVM Act does not explicitly manage risks in the areas of human and environmental health. These risk areas are now managed under the HSNO Act. The Act regulates the importation, manufacture, development, field testing, and release, of hazardous substances and new organisms in New Zealand. Under the Act a "new organism" includes an organism belonging to a species that was not present in New Zealand immediately before 29 July 1998, the date of commencement of the new organisms part of the Act. The definition includes organisms held in containment and genetically modified organisms. Although it is debatable as to whether or not the statutory definition of "substance" under the HSNO Act encompasses formulations of BCAs, ERMA has stated that it is policy for such formulations to be subject to the hazardous substance provisions of the Act. This is so that the associated risks to human and environmental health may be managed. ('Biological Preparations' in ERMA Protocol - Interpretations and Explanations of Key Concepts, No.3, Ser.2).

In summary, the marketing approval of a product which is a formulation of a BCA that is an agricultural compound will potentially require first, approval of the formulated product as a hazardous substance under the HSNO Act, second, approval for release under the HSNO Act of the BCA comprising the formulated product, and third, registration of the formulated product under the ACVM Act. Compliance with the biosecurity provisions of the Biosecurity Act 1993 would also be required for an imported product.

The requirements may at first appear onerous. However, if the product comprises a BCA which is not a new organism in New Zealand, and is classified as being exempt from registration under the ACVM Act, then the requirements for marketing approval could be limited to:

(i) ensuring that the product is manufactured in accordance with the manufacturing standard prescribed under the ACVM Act, and

(ii) assessment under the HSNO Act of the risks to human and environmental health associated with the formulated product itself.

These are matters, which a responsible manufacture of a formulation of a BCA would wish to address regardless of a regulatory requirement.

Australia

Specific requirements for biological products are included in the document *Guidelines for Registration of Biological Products*; the biological products covered fall into two broad categories; chemicals, whether or not they are biolgocially derived,and whole living organisms as microbial agents (Pitt, 1994). The overall time frame in terms of planning product launches of new products with the National Registration Scheme is 18-24 months (Taverner, 1994). The National Regulatory Authority website (http://www.dpie.gov.au/nra/bioagprod.pdf) will provide the most current advice for registration of BCAs in Australia.

U.S.A.

In the United States, a microbe used to control another microbe must be registered with the Environmental Protection Agency, whether the mode of action involves competitive displacement, anti-biotic production, or some other mechanism of control. The statutorial basis for pesticide regulation in the United States (including microbial pesticides) is The Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) and Federal Food, Drug, and Cosmetic Act (FFDCA) As Amended by the Food Quality Protection Act (FQPA) of August 3, 1996.
See the EPA web site for more details: www.epa.gov/pesticides/biopesticides

Conclusions

Biological control of deteriorated wood with microorganisms has been demonstrated in the field, around the globe. The future of this field both in terms of science and commercial benefit relies upon research to determine the conditions to give predictable results, and a variety of products on the market for various wood species. Desired results must be targeted to customer requirements. Lastly, the unique advantages the biological control agent can impart to wood properties, and the final wood product, should be further researched and developed.

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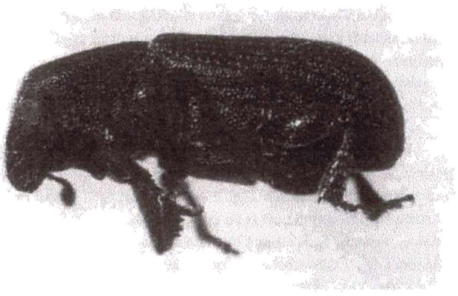
The black pine bark beetle *Hylastes ater* in New Zealand

Stephen D. Reay, Patrick J. Walsh, Joanne M. Thwaites and Roberta L. Farrell

Hylastes ater *is a European bark beetle that was first recorded in New Zealand in 1929. It is now well established in all exotic pine plantations.*

Introduction

H. ater breeds in stumps, roots and logs of pine species and is a pest in areas of second rotation forestry. The adults attack and can kill young pine seedlings following emergence from breeding material. In the past, *H. ater* has been reported as being the most troublesome insect pest in radiata regeneration, and seedling mortality of over 50% was recorded across large areas. More recently, *H. ater* has not been considered a serious forestry pest, except when occasional damage was reported.



Black pine bark beetle *Hylastes ater*

Seedlings showing evidence of severe attack by *Hylastes ater*

It is suspected that seedling mortality due to *H. ater* was often undetected in the past. Attacks on seedlings are not evenly distributed. Surveys may not cover all newly established plantings, so areas of significant mortality due to *H. ater* are often missed. *H. ater* usually attacks seedlings within the first year after planting, so surveys that are undertaken much later often fail to detect dead seedlings, as these are difficult to see. In addition, when dead seedlings are detected, the cause of death may often be contributed to other factors.

As *H. ater* attacks the root collar of seedlings, attacks cannot be detected unless seedlings are removed from the ground. For example, there were recently reports of large numbers of seedlings being killed by herbicides and drought, but on closer inspection *H. ater* was found to be the predominant cause of seedling death. Other misconceptions may have had an effect on *H. ater's* pest status. For example, *H. ater* was suggested to only attack weak or dying seedlings. There appears to be no evidence for this and large numbers of seedlings including natural regeneration were attacked in areas of low seedling mortality. However, it is likely that weak or less resistant seedlings may be more likely to die following attack. It has also been suggested that *H. ater* will only attack dead seedlings, but feeding damage is commonly observed on large numbers of living seedlings.

Despite initial concerns, *H. ater* has not been regarded as a significant forestry pest in New Zealand until recently. Recent surveys in second rotation central North Island pine forests have indicated that attacks on radiata seedlings by *H. ater* are common.

Extent of the problem due to *H. ater*

Recent seedling mortality surveys – within 1 year following planting – of 60 compartments in central North Island, planted during the winters of 1997 and 1998, showed that seedling mortality in most compartments due to *H. ater* attack was less than 5%. However, seedling mortality was higher, up to 30% in a few compartments. In addition to these mortality surveys, seedlings were destructively sampled, that is they were removed from the ground regardless of whether they were alive or dead, to observe the extent to which seedlings were being sub-lethally attacked. Interestingly, the destructive sampling revealed that seedlings in the majority of compartments were being attacked by *H. ater*, even though widespread mortality was not always observed. In fact, the incidence of sub-lethal attack was greater than 50% in two-thirds of all the compartments sampled. This indicates that a large amount of feeding damage by *H. ater* is going on undetected in these forests, and that most feeding may not result in mortality, but there are ramifications from the feeding that impact wood quality. Implications of sub-lethal attack are not yet fully understood but are of concern to foresters.

Using these surveys, and data regarding the harvesting history of these sites, it was possible to identify a relationship between site history and risk of *H. ater* damage. Seedlings planted in sites in the central North Island that had been harvested during autumn and planted the following winter were at highest risk of *H. ater* attack. This is because the peak period of *H. ater* flight activity occurs during this time and stumps in these sites are colonised by large numbers of *H. ater*. The resulting offspring develop during the following 9 to 12 months and emerge to feed on seedlings the following summer. Any seedlings planted in these sites will be attacked when these offspring emerge.

Sites harvested at other times are less likely to be severely attacked due to two main factors. Firstly, sites harvested at other times of the year are dominated by another beetle *Hylurgus ligniperda*. *Hylurgus ligniperda* is of European origin and is slightly larger but similar in appearance to *H. ater*. It became established in New Zealand in 1974 and it breeds in stumps and logs of pine species. It is commonly observed to occupy the same breeding material as *H. ater* but has not been observed to feed on seedlings. Both species may co-exist in breeding material, but one species usually dominates. As *H. ligniperda* does not attack seedlings, damage is usually slight.

Secondly, as the length of time between harvesting and planting increases, the more likely a breeding cycle of *H. ater* or *H. ligniperda* will be completed – it can be as fast as 2-3 months during summer – before seedlings are planted. Stumps appear to be unsuitable long-term breeding habitat following the emergence of beetles. Therefore seedlings planted in older harvested sites are unlikely to be attacked, as the seedlings will have been planted following the emergence of beetles.

The relationship between *H. ater* and sapstain fungi

Staining fungi are of significant economic concern to the New Zealand forest industry, due to the high susceptibility of radiata wood to staining. The invasion of wood by sapstain fungi is characterised by staining of the sapwood. This discolouration may be grey, black or brown and reflects the pigment of the fungal hyphae. While damage to the wood is suggested to be cosmetic, some species have been reported to cause a reduction in the strength of wood.

Saprophytic, pathogenic and endophytic fungi cause sapstain in wood. Saprophytic fungi are thought to be of greatest economic significance, as this group contains the most sap-staining fungal species and invades timber rapidly and prolifically after the tree is harvested. The staining effect only becomes evident when conditions are favourable for fungal growth. Staining due to pathogenic and endophytic fungi may be apparent when the tree is harvested, but these fungi are present in low concentrations in radiata pine plantations.

Many species of bark beetles including species of *Hylastes* have specialised structures, or mycangia. These are simple pits found on the head, pronotum or elytral areas that carry fungal spores. Species of *Hylastes* are known vectors of fungal root diseases in other parts of the world. In these cases, *Hylastes* adults attack the roots of stressed or diseased adult trees. During these attacks root disease fungi are transmitted to their hosts.

H. ater is a vector of sapstain fungi in second rotation radiata forests. Recently, it was found that *H. ater* carries sapstain fungi to seedlings during attacks. Sapstain fungi were isolated from surface sterilised seedlings up to six months following attack by *H. ater*. The likelihood of seedling invasion by these fungi increased with increasing severity of attack by *H. ater*. Seedlings that did not show evidence of *H. ater* attack were seldom infected with sapstain fungi. The species of sapstain that were isolated from *H. ater* and seedlings following attack by *H. ater* are given in the table. In a couple of compartments, where a substantial number of seedlings were attacked by *H. ater*, over half of the sub-lethally attacked seedlings were found to be infected by species of sapstain fungi.

This is a very significant finding and may have serious implications for radiata forestry in New Zealand. Presently, the implications of this relationship between *H. ater* feeding damage and subsequent effect on wood quality are being studied, and specifically research is currently underway to investigate this further. In particular, we are investigating the potential effects of the different species of sapstain fungi on the health of seedlings, and the long-term fate of the different species of sapstain in seedlings. It is important to understand whether sapstain fungi may have growth effects on seedlings, and if fungi are able to survive in seedlings until the tree reaches maturity, causing sapstain from the inside of the tree regardless of any treatment besides rapid kiln drying post harvesting.

Species of sapstain fungi isolated from *H. ater*, and surface sterilised seedlings following attack by *H. ater*.

Sapstain fungi isolated from *H. ater*

Ophiostoma ips
Ophiostoma setosum
Ophiostoma querci
Ophiostoma huntii
Ophiostoma galeiformis
Ophiostoma pluriannulatum
Leptographium truncatum
Leptographium procerum

Sapstain fungi isolated from surface sterilised seedlings following attack by *H. ater*

Ophiostoma galeiformis
Ophiostoma huntii
Ophiostoma setosum
Ophiostoma quercus
Ophiostoma floccosum
Ophiostoma piceae
Leptographium procerum
Leptographium truncatum

Biosecurity and bark beetles in New Zealand

The presence of the sapstain fungi *L. procerum* and *O. huntii* in New Zealand is likely to be due to their introduction with either *H. ater* or *Hylurgus ligniperda*. It is possible that the other species of sapstain fungi were introduced into New Zealand by similar pathways or on dunnage or packaged wood. The opportunity may exist for further introductions of similar species of fungi, on either *H. ater* or *H. ligniperda*, as these two species may not be subject to the same quarantine regulations as beetles not currently established in New Zealand.

Similarly, if new species of sapstain or related fungi are established in New Zealand in the future, bark beetles may be able to act as vectors. The consequences to the forest industry may be very significant if *H. ater* was able to vector fungal species such as pine pitch canker or black stain root disease throughout the forest to juvenile or mature trees. Given the high incidence of sub-lethal attack by *H. ater* in many areas, it would be necessary to re-evaluate the status of this pest if other fungal pathogens were to become established in New Zealand. In fact, *H. ater* may need to be given greater consideration as a pest of pines when the current research investigating the effects of sapstain on seedlings has been completed.

Current and future research

The sapstain group at Waikato University is currently undertaking research addressing the role of bark beetles, particularly *H. ater*, as vectors of sapstain fungi. These studies are investigating the long-term fate of sapstain fungi vectored to seedlings following sub-lethal attack by *H. ater*, and the effects of sapstain fungi on seedlings. A number of other research projects addressing other areas of bark beetle research are currently in progress. These include studies identifying characteristics that make seedlings resistant to attack, as well as management techniques to minimise damage due to *H. ater*. Finally, options for the control of *H. ater* using biological agents and insecticides are being investigated.

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Research in progress: resin degradation and brightness increase of radiata pine with fungal treatment in lab and mill trials

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Introduction

Within the last ten years two different biotechnological methods of combating resin problems in pulp and paper production were developed and are now used industrially. Hata and his colleagues of Nippon Paper Industries developed a control method using the enzyme lipase, which catalyses the hydrolysis of triglycerides. Farrell and colleagues at Sandoz Chemicals Biotech Research Corporation developed a resin degradative and biocontrol fungus for pretreatment of wood from an *Ophiostoma species* [for a review of these methods see Farrell, Hata and Wall, 1995].

The original fungal inoculum described for resin degradation, Cartapip® 97, currently marketed by AgraSol, was made by classical mating of isolated ascospores from various *Ophiostoma piliferum* isolates of the United States. *Ophiostoma piliferum* is a saprophytic Ascomycete found throughout the world and commonly referred to as one of the sapstain fungi. Sapstain fungi grow mainly in wood in the ray parenchyma cells, within resin canals, within tracheids and fiber cells and penetrate simple and bordered pits, occasionally forming bore holes through wood cell walls. Sapstain fungi are not capable of degrading cellulose or lignin but metabolise resin extractives, starch and simple sugars. Sapstain fungi cause a characteristic stain of sapwood resulting in a blue, black, grey or brown discolouration of the wood. Sapstain causes major economic losses in the lumber and some pulping industries. Problems with sapstain are most prevalent in warm, humid climates and when wood with high sapwood content is used (Kay et al. 1997).

The development of Cartapip 97 resulted from the industrial and academic collaboration with Bear Island Paper Co., a thermomechanical pulp mill in Virginia, USA which produces newsprint, the Sandoz group, and collaborators at two Universities Robert Blanchette and Thomas Harrington and their colleagues, and the sabbatical study in 1988 of Yitzhak Hadar of Hebrew University of Jerusalem. Ascospore mating and selection procedures on loblolly pine produced the isolate Cartapip 97 which grew rapidly on wood chips, degraded substantial quantities of pitch and did not stain wood (Farrell et al. 1992; Wendler et al. 1992; Hoffmann et al. 1992; Farrell et al. 1993). This colourless (albino) isolate was shown to be blocked in the 1,8-dihydroxynaphthalene (DHN) melanin synthetic pathway by the inability to produce the intermediate scytalone (Zimmerman et al. 1995). Later work showed that *O. piceae* albino strains could also be generated by the same methodology (White, 1995; White-McDougall et al. 1998). Treatments of wood chips with the albino fungi resulted in a biocontrol effect, such that the albino fungus quickly grew throughout the chips and suppressed the growth of staining fungi (Farrell et al. 1993). Biocontrol of sapstain in logs using the Cartapip 97 inoculum was demonstrated on red pine in the lab and in the field (Behrendt et al. 1995; Behrendt et al. 1995).

A four-year study concerning sapstain commenced in New Zealand in August 1996 with three objectives:

1. Broadly surveying and identifying the sapstaining organisms of New Zealand
2. Investigating the cause(s) of sapstain in radiata pine, and

   Developing an albino fungal product to control sapstain. [Preliminary work with Cartapip 97 on radiata pine had shown it was somewhat but not fully effective to reduce stain to the desired industry target of <10% stain in 3 months.]

This study was a collaboration of the similar group who made Cartapip 97, Farrell, now an academic at the University of Waikato and her colleagues at University of Waikato, Robert Hill and Stuart Kay of HortResearch of New Zealand, Blanchette and Harrington and their colleagues, and Yitzhak and Esther Hadar on sabbatical in New Zealand from mid 1996 to mid 1997, Fletcher Challenge Forests (FCF), Carter Holt Harvey Forests and New Zealand Forestry Corporation (during the study acquired by FCF) provided funding and collaboration and expertise of New Zealand plantation forestry. Clariant Corporation (formerly known as Sandoz Chemicals Corporation) contracted with the University of Waikato a license to use the intellectual property and Cartapip know-how. In conducting this research, similar to the Cartapip work, but ten years later, in a new Hemisphere, with slightly different goals, and targeted to a different wood species, the collaborators exhibited the key features for successful research, interest, collective expertise, and funding.

From the 21 sapstain fungal species isolated and identified in New Zealand (Farrell et al. 1998) four species were chosen for a breeding programme in order to make albinos. These New Zealand albinos were made as had been done with Cartapip 97, classically mating a variety of isolates of the same species to produce albino, non-melanising strains. Neither genetic engineering nor mutagens were used to make the isolates. The albino isolates have been studied for their ability to biocontrol and reduce staining in mature radiata pine logs (Farrell et al 1997a; Farrell et al 1997b), and the subject of this paper, studied for their ability to reduce resin components in radiata pine wood chips. Kimberly Clark Tantanoola Mill had investigated various methods and technologies for the decrease of resin components of radiata pine. The mill had a successful chip seasoning regime, and desired to continue seasoning chips, but in decreased time and with reliability throughout the year, regardless of chip source and weather variables. Albino fungi were studied in the lab and at the mill for the eventual goal to reduce resin (wood extractives), and improve brightness/decrease bleach chemical use after sulfite pulping. Fletcher Challenge Paper provided the expertise in wood extractives analysis to the study.

Materials and Methods

Survey, Culture Isolations and Identification, and Competition Experiments

Sampling, isolations, identifications, mating, culture growth and competition experiments on wood cubes and chips were done as described by Farrell, et al. 1998.

Lab Trial for Extractives Reduction

Fungal inocula was prepared by growth for 2 – 3 days in liquid shake cultures as described (Farrell et al 1998), harvested by centrifugation and resuspended in 10 Ms sterile water. 10e7 colony forming units of each fungal culture were applied per 100 grams dry weight wood chips (with moisture content between 45 – 55%) that had been previously sterilised in plastic resealable bags using gamma irradiation. The inoculated chips were maintained in the sterile plastic bags and incubated at 25 degrees centigrade for three weeks.

Extractives Analysis

The wood extractives content of treated and untreated chips was determined on freeze dried chip samples which had been ground to <0.5mm. The wood meal was extracted by solvent extraction with acetone using a Soxtec extractor. Gas chromatography of the methylated extract was performed using a DB-1 5m x 0.32mm capillary column fitted with a 0.5m x 0. 5mm deactivated silica retention gap and using an on column injection technique. Detection was by flame ionisation detector and quantitation was performed using the internal standard technique on the chromatography data system.

Kimberly Clark Trial Plan November, 1999 to January, 2000

The fungal inocula for the mill trial were grown as described in Farrell et al 1997.

Chips for the trial were made from freshly cut radiata pine logs. Chips were sprayed as they were conveyed toward a pile with fungal inocula. The fungal inocula were, for one pile designated OF Pile, a mixture of two *O. floccosum* albino isolates, OF 40 and OF 13 a cell concentrate sprayed in 4500 litre water solution onto 200 tons wet weight chips. It took approximately three hours to spray the piles. The other biological pile was designated OPC Pile, and consisted of a mixture of three *O. piceae* C albino isolates, OPC 580, OPC 422 and OPC 194 cell concentrate sprayed in 4500 litre water solution onto 200 tons wet weight chips.

For the mill trial, piles were made each of 200 wet weight tons of chips as follows:

DRY PILE	Chips not sprayed and put into pile
WATER PILE	Chips sprayed with water
OF PILE	Chips sprayed with water plus *O. floccosum* isolates, OF 40 and OF 13
OPC PILE	Chips sprayed with water plus OPC 580, OPC422 and OPC 194.

The piles were allowed to season in the field for 8 weeks in the field at the mill site.

Analysis of Mill Trial

Chips from each of the piles were analysed every two weeks from the 4 piles visually for stain, and biological growth. Extractives and brightness of pulps were measured.

Full extractive analyses were done on starting chips and chips taken from the four piles after 8 weeks in the field and resulting pulps from several points in the process and these results will be presented in the future.

Results and Discussion

Lab Trials for Wood Extractives Reduction

Control, non-fungal inoculated radiata pine wood chips were analysed as a reference for resin acids, fatty acids and glycerides, and the results are given in Table 1.

Although the duplicate result of the % extractives for the control appeared to be good, the duplicates on the resin acids, fatty acids and glycerides were poor. This could arise if the extract had not been dried adequately earlier in the extraction process. The poor precision found for the control made it difficult to compare with other samples. Therefore results for the fungal treated samples were compared as to the effect of one fungal isolate as compared to another fungal, rather than compared to the reference control, non-fungal treated samples.

Dozens of albino isolates were examined for their ability in the laboratory to degrade wood extractives. Results given in Table 2 show in duplicate some of the better and the lesser fungal overall effects for wood extractives decrease.

Table 1 Analysis of Wood Extractives of Control Chips from Lab Experiment

Sample		% Acetone Extractives	% Free Fatty Acids	% Resin Acids	% Glycerides
0a	Control	1.26	0.227	0.411	0.130
0b	Control	1.27	0.156	0.282	0.081
	Average:	1.26	0.19	0.35	0.11

Table 2 Analysis of Wood Extractives after Fungal Treatment

Sample	% Acetone Extractives	% Free Fatty Acids	% Resin Acids	% Glycerides	% Free Fatty Acids in Extract	% Resin Acids in Extract	% Glyceride in Extract	% Extract Identified
OPC 542	1.46	0.092	0.730	0.025	6.31	50.0	1.74	58.1
"	1.70	0.096	0.797	0.018	5.62	46.9	1.08	53.6
F63	1.19	0.108	0.481	0.020	9.11	40.4	1.72	51.2
"	1.02	0.080	0.375	0.021	7.82	36.7	2.09	46.7
7060	0.73	0.039	0.226	0.012	5.39	30.9	1.70	38.0
"	0.74	0.040	0.222	0.015	5.35	30.0	1.97	37.3
7073	0.72	0.073	0.184	0.039	10.1	25.5	5.37	41.0
"	0.68	0.069	0.168	0.036	10.2	24.8	5.25	40.2
7076	1.19	0.120	0.268	0.093	10.1	22.5	7.85	40.5
"	1.15	0.120	0.265	0.089	10.4	23.1	7.70	41.2
OPC 422	0.57	0.044	0.133	0.033	7.66	23.3	5.84	36.8
"	0.61	0.048	0.150	0.038	7.82	24.6	6.21	38.7
F13	0.62	0.036	0.100	0.016	5.82	16.2	2.52	24.5
"	0.64	0.036	0.095	0.017	5.60	14.9	2.68	23.1
OPC 580	0.65	0.038	0.165	0.010	5.79	25.4	1.61	32.8
"	0.70	0.050	0.217	0.015	7.15	31.1	2.16	40.4
F40	0.37	0.028	0.129	0.017	7.59	34.9	4.70	47.2
"	0.58	0.043	0.156	0.023	7.43	26.8	3.89	38.1

As shown in Table 2. the various isolates tested on wood chips varied considerably in their ability to degrade wood extractives. The range of extractives values for differing fungal treatments was from 0.37% to 1.70% by weight of wood: obviously some much more efficient at degrading the wood extractives than others. Fungi such as the isolate *O. floccosum* F40 on the average decreased total acetone extractives to 0.47% versus OPC 542, with the same length of time incubating on the chips. had an average acetone extractives value of 1.58% by weight of wood. Analogously. some of the fungi had significant decreases in free fatty acids. for example *O. floccosum* F40 with free fatty acids of 0.035% and free resin acids of 0.143% whereas O. floccosum isolate F63 did not decrease the free acids as much with average %free fatty acids of 0.094% and %free resin acids of 0.428% by weight of wood.

The free fatty acids and free resin acids were further analysed by gas chromatography. Examples of the data for isolate F 13 are given in Table 3.

All the data obtained to date on wood extractive decrease was sorted for the possible contenders for further evaluation on the basis that their resin acid contents were below 0.2% by weight of wood; these isolates were *O. floccosum* F40 and F13, and *O. piceae* C OPC 422, OPC 580. and OPC 194.

Species Selection for the mill trial

As the sulfite mill of interest, Kimberly Clark Tantanoola Mill is located in South Australia. a small survey of the Ophiostomataceae species was conducted at the mill site, in order to provide Australian Quarantine with relevant information as to the relatedness of the New Zealand and Australian species. From chip samples analysed in March 1999 and November 1999. the Ophiostoma species isolated and identified in South Australia - Tantanoola Mill Site were *O. floccosum*. O. *piliferum*, *O. quercus* and an unidentified Graphium sp. All taxonomic criteria were shown identical between the South Australian and the New Zealand strains.

AQIS (Australian Quarantine and Inspection Service) allowed for mill trial testing of albino isolates of *O. floccosum 40* and 13. *and O. piceae* C isolates 580. 194 and 422.

Analysis of the Mill Trial

Spray Inocula for the Mill trial

The *O. piceae* 540. *O. piceae* 422, *O. piceae* 194. culture suspensions were mixed together and applied to one pile. and *O. floccosum* 40 and *O. floccosum* 13. were mixed together and applied to another pile. as described in the Materials and Methods. From the spray tank. a sample of the inocula was taken at start. middle and end of the spraying of the 200-ton wet weight of chips, to verify the mixing and dosage of the blastospores per volume spray was consistent throughout the trial.

Fungal Growth during Eight Week Trial

Growth of the fungi at the mill piles was monitored by standard mycological culturing of chips taken from the four piles every two weeks during the trial and with some isolates checking with molecular markers for DNA analysis. Consistently. the albino *O. floccosum* grew from the OF pile. There was little if any growth from the OPC pile of *O. piceae* and there was *O. floccosum* growing in this pile. The *Wet Pile showed positive Ophiostoma identification of O. quercus, O. floccosum Rhizopus, Trichoderma, Fusarium* and yeasts. The Dry Pile showed positive Ophiostoma identification of *O. quercus, O. floccosum and Graphium species* C. *Rhizopus, Trichoderma* and other moulds were also observed.

Preliminary Pulp Analysis of the Mill trial

The wood chips of the piles were made into pulp at the mill by normal procedures. Samples of pulp were taken for further analysis from the Chemiwasher, Twin wire wash press (TWWP), and bleached pulp (M57). DNA extractions were performed on the pulps to show whether there was any trace of the New Zealand-origin fungi: these analyses showed that there was no residual DNA left in the pulps.

Soxtec dichloromethane extractions of the pulps from the chips of the 8 week mill trial (Dry pile not processed) were compared to the mill data from May 1999 to February 2000, with seasoning of chips routinely during this period being 12 - 16 weeks and results are given in Table 4.

The chips and pulps will be further analysed for their full wood extractive analysis. Brightness's of the pulps from the fungal treated pulps showed that the OPC pile pulp from the TWWP had 54.0% ISO brightness, and after bleaching 78.3% ISO brightness. The OF pile, which during the growth assays showed significant growth of the albino *O. floccosum*, and less growth of staining species showed improved brightness with the OF pulp from the TWWP having a brightness of 66.8% ISO brightness and after bleaching a 85.5% ISO brightness, a significant improvement in brightness.

Table 3 Analysis of Free acids and extractive components after F13 Lab Fungal Treatment – duplicate analysis

% Extractives	**0.62**	**0.64**
mg Analysed	0.546	0.574
C16:0 found (ug)	4.76	4.96
C18:2 found (ug)	5.94	5.51
C18:1 found (ug)	19.88	20.53
C18:0 found (ug)	4.01	4.09
Free Fatty Acids Sum (ug)	34.59	35.09
% Free Fatty Acids in Extract	6.34	6.11
Corrected % FFA in Extract	**5.82**	**5.60**
% Free Fatty Acids in Sample	0.039	0.039
Corrected % FFA in Sample	**0.036**	**0.036**
Pimaric found (ug)	15.31	14.73
Sandara. found (ug)	3.19	3.13
Isopimaric found (ug)	8.34	7.59
Levo.+Pal. found (ug)	16.53	16.02
Dehydro. found (ug)	44.26	42.96
Abietic found (ug)	2.94	3.04
Neoabietic found (ug)	5.81	5.59
Resin Acids Total (ug)	96.38	93.06
% Resin Acids in Extract	17.65	16.21
Corrected % RA in Extract	**16.20**	**14.85**
% Resin Acids in Sample	0.109	0.104
Corrected % RA in Sample	**0.100**	**0.095**
C16:0 found (ug)		
3 Unidentified (ug)	14.62	16.47
C18:1 found (ug)		
C18:2 found (ug)		
C18:0 found (ug)		
Glycerides Sum (ug)	14.62	16.47
% Glycerides in Extract	2.68	2.87
Corrected % Glyc. in Extract	**2.52**	**2.68**
% Glycerides in Sample	0.017	0.018
Corrected % Glyc. in Sample	**0.016**	**0.017**
% Extract Identified	**24.5**	**23.1**

Table 4 Wood Extractives and Brightness of Pulps from Mill (average values based on wood weight)

Sample	Chemi-washer	TWWP	M57
Mill data			
May '99– Feb 2000	0.90%	0.10%	0.07%
OF Pile pulp	0.92	0.12%	0.05%
OPC Pile pulp	0.90%	0.12%	0.06%
Wet Pile pulp	0.88%	0.10%	not done

Conclusions

This paper presents research in progress for developing albino fungi for use on radiata pine. The analyses of all the results from the first mill trial were still being completed in February 2000. The mill concluded that from the first trial even without the full analyses there were no short-term runnability issues of the fungal treated pulp. Initial indications were that the fungi have the potential to save costs at the mill by reducing bleaching chemical usage, reducing chip pile inventory and reducing wood extractives concerns. More large-scale mill trials are scheduled for year 2000. Some of the research aspects to further study include the following:

a. Why was the *O. floccosum* more successful at colonising and growing in the woodpile?
b. Will one isolate be sufficient for wood extractive decrease/brightness improvement or do multiple isolates achieve a greater effect not equaled simply by increased dosage?
c. What are the components of wood extractives in radiata pine that are most important to decrease in the pulp?

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Appendix 2 – Mass peptide peak list

Spot number	Spot 1	Spot 2	Spot 3	Spot 4	Spot 5	Spot 6	Spot 7
Gel number	A1	A2	A3	A4	A5	A6	A7
Species	*O. ips*	*O. ips*	*O. ips*	*O. ips*	*O. ips*	*O. ips*	*O. ips*
	819.2197	819.2289	803.4532	825.0646	825.2049	823.1879	825.1008
	825.2261	823.2710	819.3654	835.0611	835.1823	825.1874	835.0535
	835.1884	825.2632	823.4240	842.5101	841.2109	835.1185	842.5101
	841.2040	833.5449	825.3821	865.0112	842.6124	841.1862	865.0225
	842.6044	835.2180	832.6746	875.9280	865.1423	842.5804	886.9930
	857.2261	842.5100	835.3421	885.0343	877.1406	865.0900	918.9335
	865.1957	845.3262	841.4779	886.9587	887.1008	877.1368	926.9393
	994.2847	865.1888	845.4225	904.9689	994.2700	887.0855	1109.5710
	1015.2890	885.2268	857.3229	907.0239	1109.6355	994.1932	1197.7505
	1036.3019	916.7181	865.2972	918.8337	1197.8181	1109.5907	1211.7521
	1046.2343	994.3591	876.4265	926.9225	1245.6667	1197.7885	1245.6049
	1053.9524	1024.6746	885.2905	1940.9119	1256.7155	1211.7743	1397.7539
	1116.7726	1036.3159	994.4677	2158.9973	1541.8168	1245.6306	1541.7607
	1225.3162	1052.6431	1036.4563	2211.1045	1843.0117	1397.7527	1555.7596
	1264.1097	1053.6370	1046.4103	2225.1396	1940.9337	1541.7719	1842.9607
	1436.0049	1078.2703	1052.6948	2230.1672	2082.9954	1555.7777	1856.9652
	1440.0031	1168.8186	1054.2096	2239.2107	2159.0164	1782.9801	1940.9063
	1449.9808	1225.3164	1095.1326	2807.3506	2211.1045	1842.9799	2211.1045
	1503.0471	1264.0271	1225.4507		2225.1292	1856.9901	2239.1421
	1542.2881	1427.9158	1263.2692		2239.1704	1940.9100	2564.1387
	1602.0260	1476.0646	1267.3612		2299.1826	2211.1045	
	1665.1327	1717.0389	1304.8002		2383.9570	2225.1321	
	1794.2080	1839.0496	1478.3005		2807.2588	2239.1570	
	1923.2306	1941.2479	1906.4153			2299.1560	
	1937.2458	1994.2271	1956.2805			2564.1572	
	2211.3523	2212.3459	1970.2832			2807.2500	
	2230.4700	2239.1978	2565.3684			3347.5613	
	2308.4756	2367.6448	2807.6196				
	2675.7239	2489.2732					
	2807.6775	2547.1731					
	3338.1448	2564.3579					
	3347.9634	2606.2876					

Spot 8	Spot 9	Spot 10	Spot 11	Spot 12	Spot 13	Spot 14	Spot 15
A8	A9	A10	A11	A12	B1	B2	B3
O. ips	*O. ips*	*O. ips*	*O. ips*	*O. ips*	*O. ips*	*O. ips*	*O. ips*
825.1942	825.1298	825.1878	825.2156	825.2208	800.5350	825.2032	825.1425
835.1433	835.0766	835.1387	835.1440	835.1398	825.1944	842.6028	842.5421
865.1092	842.5270	842.6032	842.6231	842.6270	835.1536	865.1149	865.0737
887.1010	865.0441	865.1208	865.1404	865.1445	842.5941	887.0851	887.0522
905.0800	887.0200	887.0926	887.1032	887.1226	865.1219	1116.7174	905.0337
919.0411	907.1609	895.0618	895.1172	905.1309	887.0781	1396.7229	907.0248
927.0400	918.9745	905.0739	905.1197	1198.6509	895.0710	1426.7267	926.9878
1109.5710	926.9525	919.0264	919.0681	1260.7632	919.0790	1435.8224	1349.7957
1197.8867	1146.0444	927.0339	927.0172	1488.6863	927.0527	1439.8442	1363.7507
1245.7180	1154.6307	976.5876	937.0262	1566.7195	934.5362	1449.8260	1609.9476
1397.9313	1168.6627	1422.5908	1362.7896	1690.9541	997.5703	1468.7509	1631.9999
1541.9247	1205.6864	1940.8999	1422.6147	1940.9642	1198.6418	1542.0581	1663.9912
1843.1536	1221.7188	2211.1045	1438.6729	2083.0100	1488.6616	1601.8385	1693.9669
1857.1632	1809.0292	2225.1204	1915.9606	2211.1045	1562.8308	1615.8689	1841.9744
2211.2939	1835.9897	2239.1548	1940.9729	2225.1162	1690.9106	1617.8571	1940.6930
	1863.0007	2564.1550	2194.1160	2239.1313	1918.0325	1654.9581	1948.0375
	1940.9413	2807.2185	2211.1045	2283.2107	1940.9573	1664.9723	1962.0599
	2211.1489	3347.5820	2225.1062	2298.1672	2211.1045	1723.9467	1988.1975
			2410.2598	2377.2129	2377.2307	1794.0237	2103.2317
			2807.2993	2391.2087		1940.9321	2212.1655
				2564.1936		2021.0436	2226.1741
				2807.3040		2211.1045	2239.2390
				2914.4246			2564.2556
				3337.5732			
				3347.7122			

Spot 16	Spot 17	Spot 18	Spot 19	Spot 30	Spot 31	Spot 32	Spot 33
B4	B5	B6	B7	C6	C7	C8	C9
O. ips	*O. ips*	*O. ips*	*O. ips*	*O. ips*	*L. procerum*	*L. procerum*	*L. procerum*
825.1215	842.6261	835.2214	842.4980	819.2004	842.6346	842.6144	No data
842.5352	865.1478	842.6567	865.0178	825.2022	887.1242	927.0370	
865.0511	887.0920	865.1277	904.9699	835.1376	1165.6714	998.9404	
887.0057	905.0911	887.1172	926.8990	841.1956	1179.7062	1157.6917	
904.9924	919.0368	907.1572	1116.6250	865.1612	1201.7209	1179.6858	
918.9479	927.0132	919.0862	1286.7356	881.0849	1234.7893	1277.8000	
926.9370	937.0269	927.0555	1426.6884	917.4125	1277.8082	1323.7198	
936.9206	1635.7606	937.0214	1435.7335	945.6442	1365.7134	1339.7076	
977.5115	1789.9460	1997.1077	1439.7672	994.2767	1427.9081	1427.8933	
1572.7130	1803.9362	2211.1045	1449.7509	1036.3154	1475.8481	1475.8121	
1635.6678	1939.9216		1502.7604	1053.2804	1566.7964	1566.7930	
1653.8075	1997.0723		1541.8721	1118.6799	1707.8601	1716.8805	
1789.8036	2211.1045		1601.7911	1516.8809	1797.0131	1940.9073	
1803.8000			1617.8000	1552.8462	1940.9474	1994.0029	
1996.8967			1642.9156	1566.8602	1994.0278	2211.1045	
			1654.8799	1570.8364	2083.0320	2225.1523	
			1664.9170	1584.8749	2211.1045	2239.1604	
			1698.9116	1598.9264	2225.1179	2564.1169	
			1793.9651	1791.0803	2239.1245	2705.0940	
			1854.9617	1805.1281	2299.1428	2807.2397	
			1922.9041	1884.2357	2367.2661	3105.2703	
			1936.9349	1941.1310	2564.1296	3347.4602	
			2211.0728	1968.2163	2807.2661		
				2211.4202	3105.3645		
				2225.3662	3347.4297		
				2230.4575			
				2239.3767			
				2807.4380			
				3337.6956			
				3347.9758			

Spot 34	Spot 35	Spot 36	Spot 37	Spot 38	Spot 39	Spot 40	Spot 41
C10	C11	C12	D1	D2	D3	D4	D5
L. procerum	*L. procerum*	*L. procerum*	*L. procerum*	*L. procerum*	*A. alternata*	*A. alternata*	*A. alternata*
842.6462	842.6357		842.6070	842.6219	842.6649	817.9517	842.6455
927.0743	927.0337		886.0892	887.1105	858.6542	842.6270	858.6534
998.9370	957.0352		909.0697	927.0551	887.1567	858.6275	887.1210
1233.7206	1324.7477		925.0270	1277.7791	895.1032	881.5275	1234.7781
1238.8009	1338.7810		1212.6642	1324.7310	920.5637	920.5587	1277.7986
1252.8360	1408.7684		1287.6780	1340.7106	927.0857	1605.8501	1475.8381
1801.0361	1940.9502		1324.7065	1475.8019	1034.6614	1627.8394	1566.8000
1940.9222	2002.9764		1338.7386	1940.9327	1605.8680	1637.8878	1596.8669
2075.0154	2083.0225		1408.7196	2002.9519	1637.9376	1891.9871	1605.8521
2179.1641	2211.1045		1471.7770	2211.1047	1891.9995	1905.9763	1637.9030
2211.1045	2225.1377		1566.7747	2225.1265	1906.0023	2211.1045	1716.9371
2266.1177	2239.1658		1940.9430	2239.1418	1940.9746	2239.1499	1838.9927
2564.1016	2273.0334		1978.0100	2564.0908	2211.1045		1893.9769
2807.2388	2564.2002		2083.0278		2239.1411		1905.9688
3105.3831			2120.0496				1940.9561
3348.5056			2186.1785				1994.0264
			2211.1045				2010.0314
			2225.1248				2083.0217
			2246.1885				2211.1045
			2299.1770				2225.1228
			2625.3435				2239.1436
			3035.4492				2299.1482
			3346.4504				2367.2434
							2528.1951
							2564.1167
							2807.2161
							3347.3728

Spot 42	Spot 43	Spot 44	Spot 45	Spot 46	Spot 47	Spot 48	Spot 49
D6	D7	D8	D9	D10	D11	D12	E1
A. alternata	*A. alternata*	*A. alternata*	*A. alternata*	*A. alternata*	*A. alternata*	*A. alternata*	*E. nigrum*
842.6198	842.6520	817.9213	817.8978	815.9237	815.9843	842.4821	842.6138
852.5289	1297.7069	842.6498	842.6137	817.9203	817.9985	855.0297	1206.6276
877.1378	1359.6854	927.0391	927.0059	842.6384	927.0557	870.9720	1357.4307
887.1008	1362.8269	957.0369	957.0041	945.6559	957.0432	886.9446	1566.8087
1078.6117	1408.7344	996.9385	998.9706	1130.6609	998.9993	894.9301	1940.9586
1231.8157	1422.6331	998.9687	1246.5765	1198.8124	1130.6591	902.9761	2002.9406
1269.8048	1438.6567	1566.8180	1674.9026	1260.7136	1198.8215	910.9152	2083.0464
1277.7686	1484.5685	1940.9176	1940.8846	1790.9517	1790.9644	926.8871	2211.1045
1566.8079	1491.8361	2211.1045	2211.1045	1804.9629	1804.9808	1932.8617	2225.1313
1716.9049	1504.8191	2225.1323	2778.2703	1852.8818	1940.9482	1940.8566	2273.0361
1733.0186	1550.7367	2239.0962	2793.3003	1956.0366	2211.1045	2211.0310	
1773.0186	1580.8198	2299.1604		2211.1045		2383.8943	
1787.0260	1703.8456	2778.2712					
1893.9014	1915.9816	2807.2000					
1907.9395	1940.9653	3347.4192					
1940.9360	2002.9199						
1993.9948	2145.0652						
2211.1045	2194.0627						
2225.1311	2198.2756						
2239.1492	2211.1045						
2298.1975	2410.2195						
2807.2395	2473.1296						
3347.3962							

Spot 50	Spot 51	Spot 52	Spot 53	Spot 54	Spot 55	Spot 56	Spot 57
E2	E3	E4	E5	E6	E7	E8	E9
E. nigrum	*E. nigrum*	*E. nigrum*	*O. querci*	*O. querci*	*O. querci*	*O. querci*	*O. querci*
842.6353	842.6616	842.6615	842.6420	819.2464	842.5950	842.6954	842.6514
1501.7992	854.1945	887.1758	1116.7682	825.2446	861.1321	877.1932	927.0226
1566.7866	877.2079	895.1584	1426.7687	835.1978	864.5906	887.1583	957.0025
1940.9468	887.1741	917.1556	1435.8508	842.6643	885.0826	895.1287	996.8167
1987.0605	917.1932	927.0995	1439.8883	865.1970	1116.7189	917.1233	1224.6807
2002.9177	1126.6849	1501.8154	1502.9093	994.3220	1126.6021	927.0947	1427.9125
2083.0583	1282.7704	1940.9432	1541.9146	1036.3530	1148.6770	935.0593	1475.8442
2211.1045	1427.9011	2002.8984	1566.8169	1053.3430	1282.7421	957.0440	1566.8145
2225.1487	1501.8081	2083.0417	1601.8650	1116.8474	1315.7979	1326.8027	1716.9388
2230.2339	1566.7968	2211.1045	1617.8949	1225.3583	1375.6486	1403.8531	1940.9358
2239.1465	1801.0128	2225.1348	1650.9833	1436.0276	1391.6296	1791.8575	2083.0486
2272.9912	1924.9323	2239.1433	1794.0648	1440.0699	1396.6807	2211.1045	2188.0688
2284.1846	1940.9609	2273.0020	1922.9955	1476.0358	1414.7123	2383.9578	2211.1045
2298.1995	2012.0287	2807.2251	1940.9720	1503.1254	1424.7635		2225.1660
2564.1008	2083.0229		2083.0417	1542.2167	1426.6914		2519.0793
2807.2209	2211.1045		2211.1045	1602.0939	1434.7125		2564.1055
3347.4446	2225.1409		2225.1318	1618.1226	1436.7455		2782.1458
	2239.1438		2807.3389	1651.2280	1468.7111		2796.1936
	2564.1328		2883.5271	1794.3029	1541.9827		2807.2947
	2807.2805		3348.4600	1818.3234	1566.7272		3347.4375
				1923.3168	1586.7950		
				1937.3529	1601.7886		
				2211.5093	1617.8008		
				2239.5000	1640.8615		
				2330.5859	1793.9403		
				2537.6641	1940.8473		
				2564.6584			
				2675.7961			
				28.9309			
				2883.9619			
				2915.9771			
				3105.9128			
				3338.2839			
				3348.2651			

Spot 58 E10 *O. querci*	Spot 59 E11 *O. querci*	Spot 60 E12 *O. querci*	Spot 61 F1 *O. querci*	Spot 62 F2 *O. querci*	Spot 63 F3 *O. querci*	Spot 64 F4 *O. querci*	Spot 65 F5 *O. querci*
842.6472	842.6596	825.0115	816.5445	842.6379	842.6836	842.6499	819.2295
927.0388	1109.6753	834.9511	842.6227	1210.7325	1559.9047	854.2115	823.2587
957.0601	1197.8669	864.9385	917.5811	1448.8202	1902.9946	865.1953	825.2316
998.9576	1245.7128	994.0859	1090.6162	1462.8539	1940.9486	877.1977	832.4786
1088.6829	1397.8386	1036.1570	1154.6891	1510.7554	2002.9017	887.1575	835.2155
1205.7445	1541.8558	1053.9735	1168.6901	1782.9216	2083.0286	917.2159	841.2117
1256.7473	1783.0070	1179.5476	1205.7261	1796.8899	2211.1045	927.0914	867.2493
1836.9802	1843.0222	1197.6700	1216.6243	1850.9916	2225.1233	1178.7312	994.3022
1940.9537	1857.0104	1225.1062	1221.7395	1940.9382	2239.1326	2211.1045	1016.2813
2158.9670	1940.9532	1245.5176	1566.8156	2002.9286	2273.0088		1036.3279
2211.1045	2211.1045	1263.6072	1573.1342	2211.1045	2564.0813		1049.7408
2225.1162	2273.0137	1277.6608	1809.0176	2225.1406	2582.1125		1056.2788
2272.9663		1301.5255	1820.9937	2239.1611			1060.8203
		1307.6094	1831.0378	2273.0193			1094.7441
		1397.6654	1845.0458	2564.1719			1225.3333
		1475.7152	1863.0171	2807.2493			1249.3348
		1541.7040	1878.0343				1264.1031
		1650.8732	1940.9640				1436.3760
		1707.6903	2002.9675				1941.1836
		1716.8064	2083.0215				1994.2570
		1782.8958	2145.0012				2211.4336
		1792.6554	2211.1045				2230.5327
		1837.8715	2225.1343				2461.4958
		1842.9309					2549.4292
		1856.9672					2563.4941
		1890.9242					2609.5603
		1987.9902					2625.5215
		1993.9565					2807.6130
		2211.0728					
		2367.2102					
		2383.9407					
		2510.0928					
		2705.1204					
		2807.2725					
		2872.4084					
		3312.3193					

Spot 66	Spot 67	Spot 68	Spot 69	Spot 70	Spot 71	Spot 72	Spot 73
F6	F7	F8	F9	F10	F11	F12	G1
O. querci	*O. querci*	*O. querci*	*O. querci*	*O. querci*	*O. querci*	*O. querci*	*O. querci*
842.6759	842.6326	815.9725	842.6528	842.6318	817.9553	817.7108	804.5417
887.1371	957.5948	817.9703	957.0104	927.0317	842.6759	842.4540	842.6431
895.1222	1187.7119	998.9754	1174.7222	957.0441	1940.9403	1197.5284	856.6246
919.1198	1312.8085	1020.6606	1505.8031	998.9733	2002.9171	1855.7677	870.6628
927.0894	1424.8235	1392.8165	1515.8599	1077.6191	2211.1045	1940.7806	887.1589
1475.8905	1566.8037	1885.0352	1566.8345	1084.9637	2225.1362	1995.8224	927.0209
1566.8086	1940.9465	1928.9432	1671.9363	1126.7290	2272.9929	2002.7229	937.0183
1940.9850	1987.0732	1940.9492	1685.9476	1184.7616	2488.0413	2082.9568	1197.6138
2004.9994	2005.0182	2002.9233	1818.9336	1475.8396		2210.9575	1940.9344
2211.1045	2083.0613	2211.1045	1830.9210	1566.7968		2224.9924	2211.1045
	2211.1045	2225.1377	1840.9265	1940.9396		2238.9946	
	2225.1545	2230.2449	1872.9329	2003.0223		2272.8953	
	2239.1404	2273.0261	1886.9695	2083.0562		2564.0063	
	2272.9719		1940.9310	2211.1045		2625.9253	
	2283.1331		2211.1045	2225.1382		2807.0981	
	2298.1987		2225.1887	2239.1443			
	2408.0129		2273.0178	2299.1455			
	2564.1064		2383.9065	2487.9897			
	2582.1243			2564.0671			
	2678.1921			2807.2515			
	2807.2222			3347.3909			
	2914.4138						
	3105.2576						
	3347.4170						

Spot 74	Spot 75	Spot 76	Spot 77	Spot 78	Spot 79	Spot 80	Spot 81
G2	G3	G4	G5	G6	G7	G8	G9
O. querci	*O. querci*	*T. koningi*	*T. koningi*	*T. koningi*	*T. koningi*	*T. koningi*	*T. koningi*
819.2214	842.6609	842.6685	842.6510	842.6630	842.6581	842.6544	817.9658
823.2396	1566.8392	901.1301	1164.7324	1164.7312	1034.6830	927.0648	842.6620
825.2180	1919.0094	919.1051	1194.7197	1194.7388	1157.7461	936.0782	957.0035
835.5396	1934.9854	1194.6588	1255.6783	1255.6992	1350.7430	999.0172	998.9576
842.6537	1940.9473	1263.7583	1263.7855	1263.7792	1368.7637	1034.6545	1478.8666
886.3948	1987.0731	1329.7081	1325.7034	1325.7449	1382.7428	1368.7498	1491.9867
900.5134	2002.9386	1341.7306	1341.6743	1341.7330	1475.8605	1705.9617	1704.9048
917.4156	2083.0664	1357.7063	1357.7208	1357.7589	1705.9436	1873.0656	1940.9778
974.5941	2211.1045	1428.8977	1360.6914	1360.7040	1714.8905	1940.9388	2002.8896
994.2897	2225.1553	1566.8068	1372.7170	1372.7073	1873.0706	2002.9072	2211.1045
1013.6052	2239.1279	1576.0957	1428.8942	1428.8958	1940.9661	2083.0217	2273.0286
1030.5468	2273.0493	1635.8618	1521.7898	1521.7947	1971.0210	2211.1045	
1031.6172	2321.2864	1651.8818	1566.8009	1566.7976	1994.0516	2225.1389	
1038.2806	2564.1394	1698.7728	1576.0186	1576.0833	2211.1045	2239.1270	
1053.6847	2626.0596	2019.8920	1635.9045	1635.9207	2225.1409	2273.0330	
1102.6705	2807.2676		1651.9072	1651.9108	2272.9807	2807.1921	
1225.3514	3105.2998		1804.9751	1697.8727	3346.5107		
1291.8961	3347.4988		1940.9166	1804.9701			
1941.2780			2017.9259	1940.9413			
2211.4814			2031.9744	2017.9674			
2225.5234			2211.1045	2032.0105			
2231.5930				2211.1045			
2239.5464				2239.1060			
2564.6216							
2807.7563							
3105.8821							
3338.1821							
3348.2131							

Spot 82	Spot 83	Spot 84	Spot 85	Spot 86	Spot 87	Spot 88	Spot 89
G10	G11	G12	H1	H2	H3	H4	H5
T. koningi	*T. koningi*	*T. koningi*	*T. koningi*	*T. koningi*	*T. koningi*	*S. sapinea*	*S. sapinea*
815.9759	842.6479	817.9202	842.6293	842.6566	842.6393	825.2364	842.6677
817.9734	855.1990	842.6468	1212.6652	855.2057	1038.6399	865.1846	927.0875
842.6334	863.2072	865.1909	1276.6827	871.1718	1160.7092	880.5846	1187.6436
982.9743	887.1598	905.1027	1324.7291	879.1613	1190.6494	903.1245	1391.6786
988.6806	895.1498	1212.6976	1332.6830	895.1538	1212.6742	917.1220	1456.7710
1635.9523	903.1559	1276.7325	1338.7562	1212.7072	1276.7552	927.0600	1557.8156
1941.0032	917.1414	1324.7346	1340.7216	1277.7693	1314.7230	1124.7373	1777.0387
2211.1045	927.0981	1338.7644	1386.6407	1324.7444	1324.7253	1134.7062	1896.9968
2273.0313	988.6973	1408.7601	1402.6542	1338.7595	1332.7247	1461.9602	1934.0227
2807.3069	1635.9441	1940.9321	1408.7411	1340.7255	1338.7551	1475.8289	1940.9851
3348.4734	1940.8964	1977.0336	1425.7445	1408.7411	1408.7610	1485.7938	2083.0151
	2211.1045	2120.0674	1470.6721	1425.8073	1425.7721	1993.9750	2206.1377
		2211.1045	1475.8110	1475.8099	1536.8351	2127.9534	2211.1045
		2225.0000	1536.7997	1566.8162	1940.9219		2225.1201
		1245.0000	1940.9252	1940.9497	1977.0026		2239.1396
		2239.1458	1976.9817	2083.0205	2103.0449		2564.1345
		2272.9727	1993.9840	2120.0376	2120.0278		2879.3940
		2564.1028	2083.0088	2211.1045	2135.0913		
		2807.2219	2120.0273	2225.1328	2211.1045		
		3348.4385	2181.9680	2239.1511	2371.1135		
			2211.1045	2371.1321	2388.1399		
			2225.1199	2388.1228	2757.3647		
			2371.1111	2564.1453	2771.4319		
			2388.1550	2757.4458			
			2757.3809				

Spot 90	Spot 91	Spot 92	Spot 93	Spot 94	Spot 95	Spot 96	run standard
H6	H7	H8	H9	H10	H11	H12	
S. sapinea	*S. sapinea*	*S. sapinea*	*S. sapinea*	*S. sapinea*	*S. sapinea*	*S. sapinea*	
842.6787	842.6556	842.6703	842.6362	842.3917	842.6580	817.8438	804.2766
895.1080	917.1746	927.0868	993.6947	926.8365	887.1237	842.5620	819.0876
917.2726	957.0394	957.0303	1388.7723	935.7934	895.1263	917.0780	825.0953
927.0999	998.9778	998.9855	1475.8322	945.3886	927.0612	926.9544	832.3370
936.0443	1283.7311	1283.7678	1516.8594	956.7658	935.0746	945.5541	845.1436
957.0593	1566.7737	1566.8231	1716.9636	1130.4185	957.0306	1130.5951	854.3271
1475.8674	1940.9323	1794.9076	1940.9822	1198.5706	1130.6890	1500.6676	865.0240
1557.8203	2002.9288	1940.9805	2002.8912	1500.5004	1500.8003	1790.8334	876.2219
1777.0298	2211.1045	2002.8918	2083.0281	1790.6791	1790.9724	1804.8500	945.5573
1940.9824	2225.1360	2083.0222	2211.1045	1804.6840	1804.9890	1852.8412	976.4699
2002.9105	2230.2346	2211.1045	2225.1113	1940.6564	1940.9374		994.2416
2211.1045	2273.0195	2225.1345	2272.9983	1955.7552	1956.0437		1030.1180
2225.1394	2283.1855	2273.0593	2383.9678	2210.7939	2211.1045		1036.1311
2273.0459	2807.2820			2224.8254			1053.3950
				2806.9822			1132.5286
							1146.5560
							1198.6863
							1225.1198
							1262.9624
							1513.6283
							1516.6853
							1530.6571
							1786.8585
							1790.8451
							1801.8638
							1804.8715
							1818.8820
							1833.9122
							1954.0454
							1968.0028
							1981.9927
							2231.0283